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Crystallization of the N-terminal domain of human sex hormone-binding globulin, the major sex steroid carrier in blood

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The amino-teminal laminin G-like domain of human sex hormone-binding globulin (SHBG), which contains the steroid-binding site and the dimerization domain, has been produced in *Escherichia coli*, purified to homogeneity and crystallized in complex with 5α-dihydrotestosterone (DHT) in two different crystal forms. Native data sets have been collected for tetragonal crystals (space group $P4_122$ or $P4_322$; unit-cell parameters a = 52.2, c = 148.4 Å) diffracting to 3.3 Å and trigonal crystals ($R32$; a = 104.0, c = 84.4 Å) diffracting to better than 1.6 Å. Since both crystal forms can only accommodate a single monomer in the asymmetric unit and share twofold rotational symmetry, it is proposed that the homodimer of this truncated form of SHBG, as observed in ultracentrifugation experiments, displays C_2 point-group symmetry.

1. Introduction

Sex hormone-binding globulin (SHBG) is produced in the liver and circulates in blood plasma as the major sex steroid carrier protein (Hammond, 1997; Petra, 1991). It binds androgens and oestrogens with equilibrium dissociation constants in the nanomolar range (Westphal, 1986). Apart from tissue-specific glycosylation differences, SHBG is identical to the androgen-binding protein (ABP) produced by Sertoli cells (Hammond *et al.*, 1989). Plasma SHBG is a Ca^{2+}-dependent homodimeric glycoprotein and the 373 residue-long monomer consists of a repeat of two laminin G-like domains (G domains). In addition to laminin, G domains are found in a variety of proteins such as blood coagulation factor protein S, growth-arrest specific protein 6 (GAS6), crumbs and others (Joseph, 1997). There is no information about the tertiary structures of these G domains, but consensus string-sequence analysis relates them to the three-dimensional fold of the pentraxin family (Beckmann *et al.*, 1998).

Truncation experiments have shown that the steroid-binding site and main dimerization domain of human SHBG are localized within residues 1–205, which comprise the amino-terminal G domain (Hildebrand *et al.*, 1995). Furthermore, based on the observation that only one steroid molecule is bound per dimer, a model has been proposed in which the steroid is located within the dimerization interface (Petra, 1991). We have now purified and crystallized the first G domain of human SHBG (residues 1–205) in order to gain insight into the topology of G domains in general and the structural basis for sex steroid transport in blood in particular.

2. Materials and methods

2.1. Protein overproduction

Two different plasmids based on the pGEX-2T vector (Pharmacia, Uppsala) were used to produce the N-terminal domain (residues 1–205) of SHBG as such (SHBG 205) or with a C-terminal tag consisting of six histidine residues (SHBG 205-6His). In both plasmids, the human SHBG cDNA is connected to glutathione S-transferase cDNA by a linker encoding a thrombin cleavage site (Hildebrand *et al.*, 1995). Truncation of the C-terminal histidine extension present in the original construct was achieved through insertion of a stop codon after residue 205 of SHBG using the QuickChange protocol (Stratagene, La Jolla). Plasmids were introduced into *E. coli* strain JM109, which were grown in shake flasks at 310 K in LB medium (Difco, Detroit) in the presence of ampicillin (100 µg ml^{-1}) to an optical density of 0.6 at 600 nm prior to induction with 0.5 m*M* isopropyl 1-thio-β-D-galactopyranoside. Typically, after an additional 3 h of growth, about 20–25 g of cell pellet was harvested from 8 l of LB medium by centrifugation.

2.2. Protein purification

The bacterial pellets were re-suspended in 40 ml phosphate-buffered saline (PBS) containing 140 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4 pH 7.3 and 3 µ*M* DHT. After cell disruption by sonication,

Triton X-100 was added to a final concentration of 0.5% and the solution was incubated at 298 K for 30 min. The supernatant obtained after centrifugation for 1.5 h at 43 000*g* was then applied to a column containing 12 ml glutathione-Sepharose (Pharmacia) and the column was washed with 300 ml PBS buffer and 300 ml 50 m*M* Tris–HCl pH 8.5 containing 150 m*M* NaCl, 2.5 m*M* $CaCl_2$. Thrombin (Boehringer–Mannheim) was added in 12 ml of the above Tris–HCl buffer to a final concentration of 1 unit ml^{-1} and incubated with glutathione-Sepharose resin overnight at 277 K. The drained eluate and the SHBG-containing fractions obtained during subsequent washing with 30 ml Tris–HCl buffer were pooled.

The SHBG 205-6His released from the fusion protein was then further purified by affinity chromatography on a 3 ml column packed with Ni–NTA–agarose (Qiagen, Hilden), which was pre-equilibrated with buffer containing 50 m*M* Tris–HCl pH 7.5, 500 m*M* NaCl, 1 µ*M* DHT. After extensively washing the column with the same buffer, the protein was eluted with 30 ml 0.25 *M* imidazole in 50 m*M* Tris–HCl pH 7.5 containing 10 µ*M* DHT. Usually, about 3 mg of SHBG 205-6His could be obtained from an 8 l cell culture.

In the case of SHBG 205, purification was achieved using anion-exchange and gel-filtration chromatography. The cleaved protein was first applied, in the presence of 150 m*M* NaCl, to a Mono Q column (Pharmacia) equilibrated with 20 m*M* Tris–HCl pH 8.0, 2.5 m*M* $CaCl_2$, 3 µ*M* DHT. Because of the presence of salt, SHBG 205 did not bind to the column and was collected as the flow-through fraction; it was thereby separated from the major impurities. This fraction was then applied to a Superdex G75 gel-filtration column (Pharmacia) equilibrated with 20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 2.5 m*M* $CaCl_2$, 3 µ*M* DHT and eluted with the same buffer. Fractions containing pure SHBG 205 were pooled and concentrated for crystallization using Ultrafree-15 devices (Millipore, Bedford). 8 l of cell culture yielded about 10 mg of pure protein. The suitability of the protein for crystallization was confirmed using SDS–PAGE, N-terminal sequencing and circular dichroism measurements.

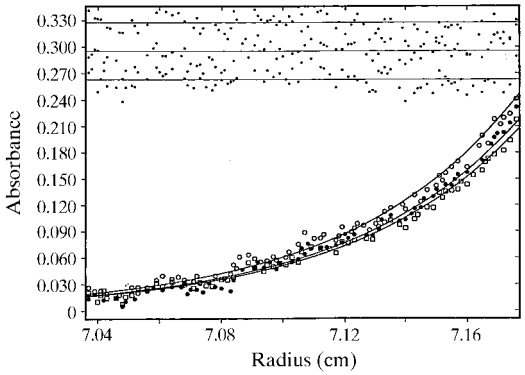

Figure 1
Radial absorbance distributions of SHBG (loading concentration: 0.2 mg ml^{-1}) at sedimentation equilibrium. The data recorded at 275 nm (open circles), 280 nm (filled circles) and 285 nm (squares) were fitted simultaneously using the program *POLYMOLE* (Behlke *et al.*, 1997), resulting in a molecular mass of 45.1 ± 1.1 kDa. Residuals (above) are given in twofold amplification.

2.3. Ultracentrifugation experiments

Sedimentation equilibrium experiments were performed in a XL-A-type analytical ultracentrifuge (Beckman, Palo Alto). About 70 µl of SHBG 205 protein solution (loading concentration 0.2–0.4 mg ml^{-1} in 50 m*M* Na HEPES buffer pH 7.5, 2.5 m*M* $CaCl_2$, 3 µ*M* DHT) was centrifuged in standard double-sector cells with a 12 mm optical path for 2 h at 26 000 rev min^{-1} (overspeed) and then for 20 h at 22 000 rev min^{-1} and 293 K. From the radial absorbance distributions at sedimentation equilibrium (recorded at 275, 280 and 285 nm), the molecular mass of SHBG 205 was calculated to be 45.1 ± 1.1 kDa (Fig. 1) with the program *POLYMOLE* (Behlke *et al.*, 1997).

2.4. Crystallization

Initial crystals of SHBG 205-6His were obtained using the sparse-matrix screening approach (Jancarik & Kim, 1991). Excessive precipitation of protein during crystallization trials was avoided by substituting the Tris–HCl buffer in the protein solution by an imidazole–HCl buffer. Crystals of form *A* were grown using the hanging-drop method at 277 K. This was performed by the equilibration of a droplet prepared by mixing 1 µl of protein solution (13 mg ml^{-1} protein, 80 m*M* imidazole–HCl pH 7.5, 1 m*M* $CaCl_2$, 10 µ*M* DHT) with 1 µl reservoir solution (10% 2-propanol, 20% PEG 4000 in 100 m*M* Na HEPES pH 7.5) against 1 ml of reservoir solution (Fig. 2*a*). Crystals grew within three months to final dimensions of approximately 200 × 100 × 50 µm.

A second crystal form (form *B*; Fig. 2*b*) was first observed for SHBG 205 and could later be reproduced with SHBG 205-6His. These crystals grew within one week at 293 K, after mixing 1 µl of protein solution (13 mg ml^{-1} protein, 50 m*M* Na HEPES pH 7.5, 2.5 m*M* $CaCl_2$, 3 µ*M* DHT) with 1 µl of reservoir solution (20% 2-propanol, 10% PEG 400 in 100 m*M* Na HEPES pH 7.5), and by equilibrating this against 1 ml of reservoir solution.

2.5. Preliminary crystallographic analysis

Both crystal forms can be flash-frozen in liquid nitrogen prior to X-ray exposure. In the case of form *B* no additive is needed, while crystals of form *A* have to be soaked for 30 s in reservoir solution containing 10% glycerol. A native data set was collected for form *A* using an image plate (MAR Research, Hamburg) mounted on a rotating-anode generator using the oscillation method and Cu *Kα* radiation. The crystals belong to space group $P4_122$ or $P4_322$, with unit-cell dimensions $a = 52.2$, $c = 148.4$ Å. In the resolution range 20–3.2 Å, the data set is 98.7% complete with an overall R_{merge} of

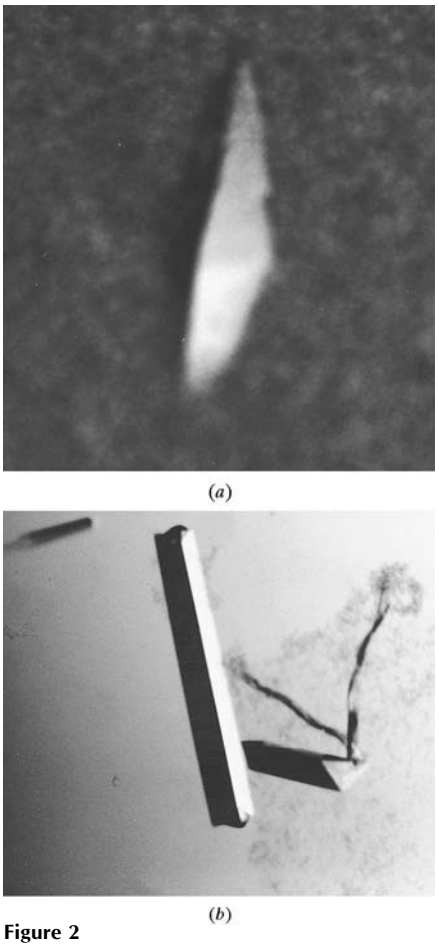

Figure 2
(*a*) Crystal form *A* of SHBG 205-6His in space group $P4_122$ or $P4_322$, with approximate dimensions 200 × 100 × 50 µm. (*b*) Crystal form *B* grown from SHBG 205 in space group *R*32, with approximate dimensions 100 × 100 × 700 µm. In contrast to crystal form *A*, crystals of form *B* can be rapidly reproduced and diffract to higher resolution.

14.5%. Assuming one molecule in the asymmetric unit, the solvent content is 41% (Matthews parameter V_M = 2.1 Å^3 Da^{-1}).

A high-resolution native data set to 1.55 Å resolution was collected from crystal form *B* on beamline X11 at the EMBL outstation of the DESY synchrotron in Hamburg. The crystals belong to space group *R*32, with unit-cell dimensions a = 104.0, c = 84.4 Å. The data set collected at a wavelength of 0.9119 Å is 96.4% complete (95.0% in the resolution range 1.75–1.55 Å). $R_{\rm merge}$ is 4.2% for all data and 23.4% in the highest resolution shell. The solvent content is as low as 35.5% (V_M = 1.9 Å^3 Da^{-1}). Both data sets were processed with the program *XDS* (Kabsch, 1988).

3. Results and discussion

We have succeeded in the production and subsequent crystallization, in two different crystal forms, of the amino-terminal laminin G-like domain of human SHBG. For this purpose, a standard glutathione S-transferase (GST) fusion system could be used, making it unnecessary to target SHBG into the periplasma of *E. coli* by inclusion of a signalling sequence. The resulting product is fully active in terms of steroid binding (Hildebrand *et al.*, 1995). The fact that this domain of SHBG and glutathione S-transferase are both capable of forming homodimers in solution did not negatively affect protein production or purification. Protein loss through formation of higher molecular weight polymers and subsequent precipitation was not apparent.

We focused initially on the production of SHBG 205-6His, which includes a His-tag at the C-terminus. This construct has the advantage that besides using Ni-specific affinity chromatography during purification, the C-terminal location of the His-tag helps to eliminate protein heterogeneity resulting from early termination events during protein translation. The protein obtained in this way was more than 99% pure as judged by SDS–PAGE. However, the protein tended to aggregate following thrombin cleavage. This could be prevented when imidazole buffer was used for protein handling, hinting that the His-tag might contribute to protein aggregation. Removal of the His-tag led to a threefold to fourfold increase in yields and a remarkable increase in the solubility of the protein.

Of the two crystal forms, form *B* is better suited for structure determination. The crystals grow faster, are easier to reproduce and diffract to higher resolution. Initial phases were recently obtained from a *cis*-$Pt(NH_3)_2Cl_2$ derivative of form *B*. Comparison of the observed mass of 45.1 kDa obtained in the ultracentrifugation experiment with the calculated mass of 22.7 kDa of the monomer indicates that SHBG 205 is homodimeric in solution. However, in both crystal forms only a single monomer can be accommodated in the asymmetric unit, as both crystal forms are characterized by low solvent contents. The only symmetry element common to both crystal forms is the twofold symmetry; we expect the SHBG homodimer to display C_2 point-group symmetry.

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