2. Materials and methods

2.1. Preparation of competent *E.coli* cells

JM109 cells were made competent following the Inoue method (Inoue *et al.*, 1990). A single *E.coli* colony was picked up from a LB plate and transferred to 25 ml of LB medium. After incubation at 37° C overnight, 100 µl of preculture was added to 25 ml of LB medium and grown to an optical density of 0.5-0.6 at 600 nm . Then the culture was quickly transferred on ice for 10 minutes and the cells were harvested by centrifugation at 2500 g in a Heraeus 28 RS centrifuge for 10 minutes at 4° C. The cell pellet was resuspended in 8 ml of TB buffer containing 10 mM PIPES (piperazine-1,2-bis[2-ethanesulfonic acid]), 15 mM $CaCl_2$, 250 mM KCl and 55 mM $MnCl_2$ pH 6.7 and incubated on ice for 10 minutes. Following another centrifugation step (4° C, 2500 g, 10 minutes), cells were resuspended in 2 ml of TB buffer, 140 µl DMSO (dimethylsulfoxide) was added and the bacterial suspension was stored on ice for 10 minutes. Then it was aliquoted in 200 µl samples in 1.5 ml sample tubes. The aliquots were quickly frozen in liquid nitrogen and stored at -70° C.

2.2. Transformation

Frozen aliquots of competent *E.coli* cells were thawed on ice and 1 µl of plasmid (10-30 ng/µl) added. Gently mixed, the cells were stored on ice for 30 minutes and then incubated at 42° C for 45 seconds. After heat shock incubation the cells were rapidly transferred on ice and stored for 2 minutes. Then 1ml of LB medium was added and the culture incubated at 37° C for 1 hour to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. 100 µl of transformed competent cells were transferred onto LB plates containing the appropriate antibiotic (Inoue *et al.*, 1990). Plates were placed at 37° C for 12-16 hours until colonies appeared.

2.3. Protein production

Two different plasmids based on the pGEX-2T vector (Pharmacia, Uppsala, Sweden) were used to produce the N-terminal LG domain of SHBG (residues 1 to 205) either without (SHBG 205) or with a C-terminal tag consisting of six histidine residues (SHBG205-6His). The plasmid containing SHBG205-6His was obtained from Prof. G. Hammond. In both plasmids, human SHBG cDNA is fused to glutathion S-transferase cDNA by a linker encoding for a thrombin cleavage site (Hildebrand *et al*., 1995). Truncation of the C-terminal histidine extension present in the original construct was achieved through the insertion of a STOP codon after residue 205 of SHBG using the QuickChange protocol (Stratagene, La Jolla, CA). Both plasmids were transformed into *E. coli* strain JM109, and the cells were grown in shake flasks at 37°C in LB medium (Difco, Starks, USA) in the presence of ampicillin (100 μg/ml) to an optical density of 0.6 at 600 nm prior to the induction with 1 mM isopropyl-1-thio-β-D galactopyranoside (IPTG). Typically, after three additional hours of growth, about 20 – 25 g of cell pellet could be harvested from 8 l of LB medium by centrifugation.

2.4. Protein purification

The bacterial pellets were resuspended in 40 ml of phosphate-buffered saline (PBS) containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.3 and 3μM DHT. The suspension is sonicated while cooling in an ice water bath to disrupt the bacterial cells. Triton X-100 was added to a final concentration of 0.5 % and the solution was incubated at 25° C for 30 min. The filtrated supernatant obtained after centrifugation in a Beckman J2-MC centrifuge with JA-20 rotor at 43 000 g for 1.5 h at 4° C was then loaded on a column containing 12 ml of glutathione-sepharose (Pharmacia), and the column was washed with 300 ml PBS buffer and 300 ml 50 mM Tris-HCl, pH 8.5, containing 150 mM NaCl, 2.5 mM $CaCl_2$. Thrombin (Boehringer-Mannheim, Mannheim, Germany) at a final concentration of 1 unit/ml and 50 μg DHT were added in 12 ml of the above Tris-HCl buffer to the glutathione-sepharose resin. The mixture was incubated at the rotating platform overnight at

4° C. The drained eluate as well as SHBG-containing fractions obtained during subsequent washing with 30 ml of Tris-HCl buffer were pooled.

SHBG205-6His released from the fusion protein was then further purified by affinity chromatography. The protein was loaded onto a 3-ml column packed with Ni-NTA-agarose (Qiagen, Hilden, Germany) pre-equilibrated with the buffer containing 50 mM Tris-HCl, 500 mM NaCl, 3μM DHT pH 7.5 and after extensive washing with this buffer the protein was eluted with 30 ml 0.25 M imidazole in 50 mM Tris-HCl, pH 7.5, containing 10 μM DHT. Usually about 3 mg of SHBG205-6His could be obtained from 8 l cell culture.

In case of SHBG205 without a His-tag, purification was achieved using anion-exchange and gel-filtration chromatography. The fraction containing the protein in PBS buffer obtained after affinity chromatography on glutathione-sepharose resin was first applied on a Mono Q column HR 5/5 (V=1 ml) (Pharmacia) equilibrated with 5 ml buffer containing 150 mM NaCl, 20 mM Tris-HCl, 2.5 mM $CaCl_2$, 3 μM DHT pH 8.0. Because of the presence of the salt, SHBG205, in contrast to major impurities, did not bind to the column and was collected as the flow-through fraction. This fraction has been concentrated from 50 to 1.5 ml using Ultrafree-15 devices (Millipore, Bedford, USA) in a Heraeus 28 RS centrifuge equipped with the rotor HFA 5.5 at 2 000 g for about 2 h at 20° C. Concentrated protein solution was then applied on a Superdex G75 16/60 gel filtration column (V=125 ml) (Pharmacia) equilibrated with 250 ml buffer containing 20 mM Tris-HCl, 100 mM NaCl, 2.5 mM $CaCl_2$, 3 μM DHT pH 8.0 and eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions with a volume of 1.5 ml were collected. Fractions containing pure SHBG205 were pooled, dialysed against buffer containing 50 mM Na-HEPES, 2.5 mM $CaCl_2$, 3 μM DHT pH 7.5 and concentrated for crystallization experiments to a final protein concentration of 13 mg/ml using Ultrafree-15 devices (Millipore, Bedford) Heraeus 28 RS centrifuge in rotor HFA 5.5 at 2 000 g for about 1 h at 20° C. Eight litres of cell culture yielded about 10 mg of pure protein. Crystallization suitability of the protein was confirmed using SDS-PAGE electrophoresis, N-terminal sequencing and circular dichroism measurements.

Purification of SHBG205 in complex with different ligands, namely, estradiol, 5α-androstane,3β,17β-diol (17β-dihydroandrosterone (17β–DHA)), 5α-androstane,3β,17α-diol (17α–DHA), methoxyestradiol and norgestrel, was achieved by using the same procedure as

described for 5α-DHT, except that the corresponding ligands were used instead of DHT during all steps of the purification procedure. Estradiol and norgestrel were purchased from Sigma, St. Louis, MO, all other ligands were from Steraloids, Newport, USA.

2.5. Polyacrylamide gel electrophoresis

To check the quality of the protein conventional one-dimensional SDS-PAGE was performed according to standard protocols (Laemmli, 1970) with some modifications. The protein samples (1-5 µg of the protein in buffer containing 0.5 M Tris-HCl, pH 6.8, 10 % glycerol, 10 % SDS, 5 % 2-mercaptoethanol) were subjected to glass plates of denaturing SDS-PAGE containing 1.5 ml of stacking gel: 5 % gel (0.25 ml of stock solution: 30 % acrylamide: 0.8 % methylenebisacrylamide), 0.125 M Tris-HCl, pH 6.8, 0.1 % SDS, 0.05 % ammonium persulfate (APS), 0.1 % TEMED and 5 ml of resolving gel: 15 % gel, 0.375 M Tris-HCl, pH 8.8, 0.1 % SDS, 0.05 % APS and 0.05 % TEMED using 250 ml of electrode buffer containing 0.25 M Tris, 1.9 M glycine, 0.2 % SDS pH 8.3. The electrophoresis was performed in a Mini-Protean II electrophoresis Cell (Bio-Rad, Hercules, USA) at 180 mV for 1 hour. After electrophoresis, the gel was bathed in a solution containing 0.1 % Coomassie Brilliant Blue G-250, 40 % methanol, 10 % acetic acid for 30 minutes and subsequently washed with a solution containing 10 % methanol and 5 % acetic acid.

2.6. Crystallization

2.6.1. SHBG in complex with DHT

Two different crystal forms were obtained. Initial crystals of SHBG205-6His were obtained using the sparse matrix screening approach (Jancarik *et al.*, 1991). Crystals in the tetragonal and trigonal forms were grown using the hanging drop method (**Figure 2.1**). In this method, drops are prepared on a siliconized microscope glass cover slip. The slip is placed above the precipitant solution and is sealed off by grease. Equilibrium is reached by diffusion of vapor from the drop to the precipitating solution or vice versa. Crystals grew within 3 months at 4°C after equilibrating a droplet prepared by mixing 1µl of protein solution (13 mg/ml protein, 80

mM imidazol-HCl, pH 7.5, 1 mM CaCl2, 10 µM DHT) with 1 µl reservoir solution (10 % isopropanol, 20% PEG 4000 in 100 mM Na-HEPES pH 7.5) against 1 ml of reservoir solution. Crystals have final dimensions of about 200 x 100 x 50 μm^3.

The trigonal crystal form was first observed for SHBG205 and could later be reproduced with SHBG205-6His. These crystals grew within one week at 20°C after mixing 1 µl of protein solution (13 mg/ml protein, 50 mM Na-HEPES, pH 7.5, 2.5 mM $CaCl_2$, 3 µM DHT) with 1 µl of reservoir solution (20% isopropanol, 10% PEG 400 in 100 mM Na-HEPES, pH 7.5) and equilibrating against 1 ml of reservoir solution.

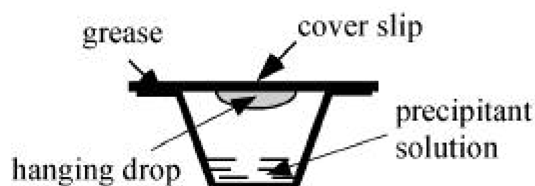

Figure 2.1 The hanging drop method of protein crystallization.

2.6.2. Crystallization of different SHBG-ligand complexes

SHBG205 in complex with different ligands, such as estradiol, 17β-DHA, 17α-DHA, methoxyestradiol and norgestrel, was crystallized reproducing the conditions used for the growth of the trigonal crystal form, except that the corresponding ligand was used instead of DHT. Estradiol, 5α-androstane,3β,17β-diol (17β-dihydroandrosterone (17β–DHA)), 5α-androstane,3β,17α-diol (17α–DHA) were used like DHT at a 3 µM concentration while methoxyestradiol and norgestrel were used in 10 µM concentrations. In case of SHBG in

complex with estradiol, 17α–DHA and methoxyestradiol, 100 mM $CaCl_2$ was added to the reservoir solution.

2.7. Ultracentrifugation experiments

In biological systems SHBG exists as a homodimer. To test whether dimerization also occurs in the crystallization buffer, ultracentrifugation experiments were performed. Sedimentation equilibrium experiments were performed in a XL-A type analytical ultracentrifuge (Beckman, Palo Alto). About 70 µl of SHBG205 protein solution (loading concentration 0.2- 0.4 mg/ml in 50 mM Na-HEPES buffer pH 7.5, 2.5 mM $CaCl_2$, 3 µM DHT) was centrifuged in standard double sector cells, with a 12 mm optical path, for 2 h at 26000 rpm (overspeed), and then for 20 h at 22000 rpm and 20°C. The molecular mass of the protein was 45.1 ± 1.1 kDa and corresponded to a dimer of SHBG205 as calculated from the radial absorbance distributions at sedimentation equilibrium (recorded at 275, 280 and 285 nm) with the program POLYMOLE (Behlke *et al*., 1997).

2.8. X-Ray diffraction experiments

All crystals were mounted in cryo loops and flash frozen in liquid nitrogen prior to X-ray exposure. Whereas in case of the crystals of SHBG205 in complex with DHT no additive was needed, the tetragonal crystals had to be soaked for 30 seconds in a reservoir solution to which 10 % glycerol was added. For the later data collection of different complexes of SHBG205, cryo conditions were optimized and the crystals soaked in solutions containing 30 % isopropanol, 10 % PEG 400 in 100 mM Na-HEPES, pH 7.5. All datasets were collected at 100 K.

Four different derivatives were prepared to solve the native structure. Trigonal crystals of SHBG205 in complex with DHT were soaked in heavy atom solutions. The native data set (103 frames with a 0.5° oscillation width) and the $PtCl_4$ data set were collected at beam line X11 of the EMBL outstation at DESY synchrotron in Hamburg at the fixed wavelength of 0.84 Å using a MAR CCD 165 mm detector system. The remaining derivatives were collected using a Rigaku RU H2R direct drive rotating anode generator producing CuKα radiation

(**Table 2.1**). The generator was run at 44 kV, 110 mA with a 0.3 mm focus. Data was collected with a 30 cm MarResearch image plate (Hamburg, Germany). All data sets were reduced with the program XDS (Kabsch, 1988).

A 1.7 Å resolution data set of the EDTA soaked crystals and a dataset to 2.35 Å of the tetragonal crystals were collected on beamline ID14 at the ESRF synchrotron in Grenoble at a wavelength of 0.933 Å using a MAR CCD 165 mm detector system (**Table 2.2**). In case of the EDTA soaked crystals, a 1.7 Å high resolution data set consisting of 60 images with an oscillation range of 1° and a 2.45 Å low resolution data set consisting of 30 images with an oscillation range of 2° have been collected and merged together. In case of the tetragonal crystals, 110 images with an oscillation range of 1° were collected. Again, all the data sets were reduced with the program XDS (Kabsch, 1988).

A 1.8 Å resolution data set of the crystals of SHBG in complex with estradiol were collected at beam line BW7A at the EMBL outstation at the DESY synchrotron in Hamburg using synchrotron radiation at a wavelength of 0.9102 Å and a MAR CCD 165 mm detector system (**Table 2.3**). A set of 121 images was collected with a 0.5° oscillation range. The data sets of the crystals of SHBG in complex with 17α-DHA (75 images with 0.6° and 31 images with an oscillation range of 1.5°), norgestrel (70 high resolution images with 0.6° and 23 images with an oscillation range of 1.5°) and methoxyestradiol (86 images with 0.6° and 24 images with an oscillation range of 2.0°) were collected at beam line BW7B of the EMBL outstation at the DESY synchrotron in Hamburg and a wavelength of 0.8453 Å using a MAR IP 345 mm detector system (**Table 2.3**). The datasets were reduced with the program package DENZO/SCALEPACK (Otwinowski and Minor, 1997). A 2.0 Å resolution data set of SHBG in complex with 17β-DHA was collected using CuKα radiation from the in-house rotating anode source (**Table 2.3**) and reduced with the program XDS (Kabsch, 1988).

Table 2.1 Summary of the X-ray data collection for the original crystal structure of SHBG.

Data collection and MIRAS analysis	Native	$PtCl_4$	*cis*-$Pt(NH_3)_2Cl_2$	K_2PtCl_6	$GdCl_3$
Soaking concentrations (mM)/ time (h)	-	5 / 72	5 / 12	5/12	10 / 72
Radiation source	X11 Hamburg, DESY	X11 Hamburg, DESY	rotating anode MDC	rotating anode MDC	rotating anode MDC
Wavelength (Å)	0.9119	1.072	1.5418	1.5418	1.5418
Resolution (Å) *	1.55 (1.75 - 1.55)	2.55 (2.6 - 2.55)	2.80 (3.0 - 2.8)	2.80 (3.0 - 2.8)	4.0 (4.45-4)
Completeness (%) *	96.4 (95.0)	94.3 (32.8)	84.9 (81.6)	99.2 (99.6)	91.7 (79.2)
Completeness of anomalous difference	-	93.4	71.3	-	-
R_{MERGE} (%) *, #	4.2 (23.4)	5.2 (33.6)	7.0 (17.0)	7.4 (19.1)	7.6 (7.9)
$<I/\sigma>$ *	16.5 (3.35)	9.36 (2.16)	9.93 (4.47)	14.4 (5.14)	11.8(9.82)
R_{ISO} (%) +	-	21.0	20.2	17.1	19.6
Number of heavy atom sites	-	6	3	3	1
R_{CULLIS} (%) §	-	66	62	71	81
Phasing power $	-	1.85	2.09	1.58	1.26

* Resolution limits and values for outer resolution bins are given in parentheses; # $R_{MERGE} = \Sigma\Sigma|I_i - <I>|/\Sigma\Sigma I_i$; + $R_{ISO} = \Sigma| |F_P|-|F_{PH}|| / \Sigma |F_P|$; § $R_{CULLIS} = \Sigma| |F_{PH}\pm F_P|-F_H| / \Sigma |F_{PH}\pm F_P|$, F_P, F_{PH}, F_H being the structure factor amplitudes of the native protein, the heavy atom derivative and the heavy atom contribution respectively; $ Phasing power = $<|F_H|> / <IE>$, $<IE>$ being the root-mean-square lack of closure error

Table 2.2 Summary of the X-ray data collection for the tetragonal and EDTA-soaked crystals.

	Trigonal crystals EDTA-soaked	Tetragonal crystal
Data collection		
Source	ID14 Grenoble, ESRF	ID14
Space group	R32	$P4_322$
Cell parameters: *a, c* (Å)	103.72, 85.76	52.24,148.53
Resolution (Å)	40-1.7 (1.8 – 1.7)[a]	40-2.35 (2.4 – 2.35)[a]
Number of reflections	90466	72753
Unique reflections	19262	8915
Completeness (%)	98.1 (99.2)	94.9 (99.1)
R_{merge}(%)	6.2 (37)	9.1 (37.9)

[a] Values for the highest resolution shell are given in parentheses.

Table 2.3 Summary of X-ray data collection of different steroid complexes of SHBG.

	SHBG-ligand complexes				
	estradiol	17α–DHA	norgestrel	methoxyestradiol	17β–DHA
Source	BW7A	BW7B	BW7B	BW7B	rotating anode
		Hamburg, DESY			MDC
Resolution (Å)	20-1.8 (1.82-1.8)	20-2.0 (2.03-2.0)	20-2.0 (2.03-2.0)	20-1.75 (1.79-1.75)	20-2.0 (2.03-2.0)
Space group	R32	R32	R32	R32	R32
Cell parameters					
a,b	103.63, 84.49	103.9, 84.48	103.98, 83.93	103.92, 84.46	103.89, 84.58
Number of reflections	159431	105549	80449	123818	39305
Unique reflections	15751	11516	11554	17701	11464
Completeness (%) (overall/outer shell)	96.6/70.4	96.1/94.3	97/82.6	99.4/96.9	95.4/93.7
R_{sym} (%)	3.2/28.0	2.8/12.5	3.8/29.9	3.1/28.6	4.3/23.6

[a] Values for the highest resolution shell are given in parentheses.

2.9. Structure determination

2.9.1. Solving the "original" structure of SHBG

The crystal structure of SHBG was first solved in complex with DHT. This structure is in the following referred to as the "original" structure of SHBG. The structure was solved using the trigonal crystal form with the multiple isomorphous replacement method (M.I.R.). This method requires several steps. The heavy atom positions were detected with the program HEAVY (Terwilliger, 1987) and refined with the program MLPHARE from the CCP4 program suite (CCP4, 1994). Anomalous differences from two of the derivatives were included in phase calculations resulting in an overall figure of merit of 0.56. After solvent flattening, visual inspection of the electron density at 2.6 Å resolution revealed most of the chain fold, as well as the position of the steroid. Because of the high resolution of the native data, experimental phases could be automatically extended to 1.55Å using the free atom refinement method in the program ARP/wARP (Perrakis *et al.*, 1997). Automatic chain tracing and subsequent sequence docking resulted in a model consisting of 122 out of 205 residues. Of those 52 % were modeled according to their correct sequence while the remaining ones were modeled as alanines or serines. The model was then manually completed with the program O (Jones *et al.*, 1991) and subjected to several cycles of refinement with the program REFMAC (CCP4, 1994) and manual rebuilding. Water molecules in the final model were located by the following criteria: appearance of the density in $2F_o$-F_c maps contoured at more than 1σ, distances between water molecules and neighboring atoms greater than 2.5 Å and refined temperature factors being less than 60 $Å^2$.

2.9.2. Solving the crystal structure of SHBG in the tetragonal crystal form

The structure of SHBG in the tetragonal crystal form was solved by molecular replacement with the program AMORE (CCP4, 1994) using the "original" crystal structure as a search model. Eventually, the positional parameters were optimized by rigid-body refinement (Navaza, 1994).

The tetragonal crystals contain one monomer in the asymmetric unit. The rotation function calculated in the resolution range between 10 and 4 Å yielded an unambiguous solution with a correlation coefficient of 0.32 compared to the highest noise peak at 0.20. The translation function yielded a correlation coefficient of 59.7 and a R-factor of 40.4 %. Rigid-body refinement improved this solution to a correlation coefficient of 59.9 and a R-factor of 39.8 %. The loop segment from Pro130 to Arg135, covering the steroid-binding pocket, became clearly visible on a Sigmaa-weighted difference Fourier electron density map (Read, 1986). Refinement to convergence was performed with the program REFMAC (CCP4, 1994). In the final stages of refinement, an explicit bulk-solvent mask was calculated with the program XPLOR (Brunger *et al*., 1998) and introduced into REFMAC.

2.9.3. Solving the crystal structure of SHBG in the EDTA-soaked crystals

To determine the crystal structure in the absence of any contaminations from divalent ions, trigonal crystals of SHBG205-6His were harvested and soaked for three days in a solution containing 10 mM EDTA, 5% PEG 400, 20% isopropanol, 100 mM Na-HEPES pH 7.5.
The structure of the EDTA-soaked trigonal crystals was solved by difference Fourier techniques using the protein atomic coordinates for SHBG in complex with DHT from the "original" crystal structure. The previously missing loop segment was clearly visible in the electron density maps and could be built with confidence with the program O (Jones *et al*., 1991). The model was subjected to several rounds of refinement with the program REFMAC alternated with manual inspection in the program O (Jones *et al*., 1991). Here again, in the final refinement rounds, low resolution data were corrected by the introduction of an explicit bulk-solvent mask.

2.9.4. Solving the crystal structures of various SHBG-ligand complexes

The crystal structures of SHBG-ligand complexes were solved by difference Fourier techniques using the protein atomic coordinates for SHBG in complex with DHT from the "original" crystal structure.

The coordinates of DHT, estradiol, 17β-DHA, norgestrel, 11-methoxyestradiol were obtained from the Cambridge small molecule structural database. 2-methoxyestradiol was constructed by taking the A-ring from the 2,3-dimethoxy-derivative of estradiol and combining with estradiol. 17α-DHA was modeled from 17α−estradiol using the program O (Jones *et al.*, 1991). To include these ligands in the refinement, separate topology and parameter files for each of the ligands had to be constructed. The topology file contains the description of the atoms, the assignments of covalent bonds, bond angles, etc. The parameter file contains the expected values for the geometric parameters described in the topology files. Topology and parameter files for each ligand have been created using the Hic-Up server at Uppsala software factory (Kleywegt and Jones, 1998). The models of the SHBG-ligand complexes were subjected to several rounds of refinement by molecular dynamics and energy minimization using the program CNS (Brunger *et al.*, 1998) alternated with manual inspection in the program O (Jones *et al.*, 1991).