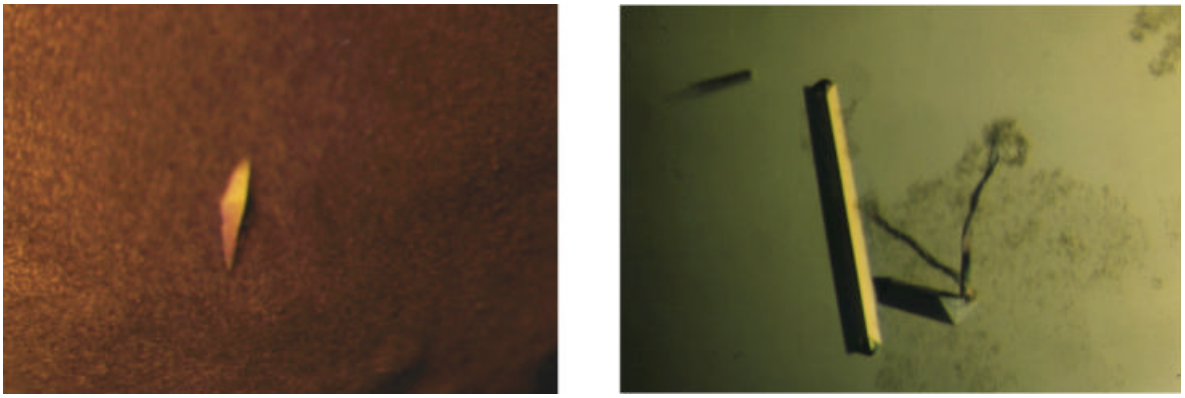
3. Results

3.1. Crystal structure of the N-terminal domain of human SHBG in complex with DHT

3.1.1. Crystallization

For crystallization experiments the amino-terminal laminin G-like domain of human SHBG (SHBG) was overproduced and purified to homogeneity. For this purpose, a standard glutathion S-transferase (GST) fusion system could be used. We focused initially on the production of SHBG205-6His, 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 3 to 4 fold increase in yields and a remarkable increase in solubility of the protein in the absence of imidazole.

The purified SHBG was subsequently crystallized and two different crystal forms were obtained. Of the two crystal forms (tetragonal crystals (form A) and trigonal crystals (form B)) (**Figure 3.1**), form B was better suitable for the structure determination. The crystals grew faster, they were easier to reproduce, and diffracted to higher resolution. In both crystal forms only a single monomer is accommodated in the asymmetric unit. Both crystal forms are characterised by low solvent contents: 41 % (Matthews parameter: V_M = 2.1 Å^3/Da) for the crystal form A and 35.5 % (V_M= 1.9 Å^3/Da) for the form B.



(a) (b)

Figure 3.1 (a) Crystal form A of SHBG205-6His in space group $P4_322$, with approximate dimensions 200×100×50 μm^3. (b) Crystal form B grown from SHBG 205 in space group R32, with approximate dimensions 100×100×700 μm^3.

3.1.2. Structure refinement

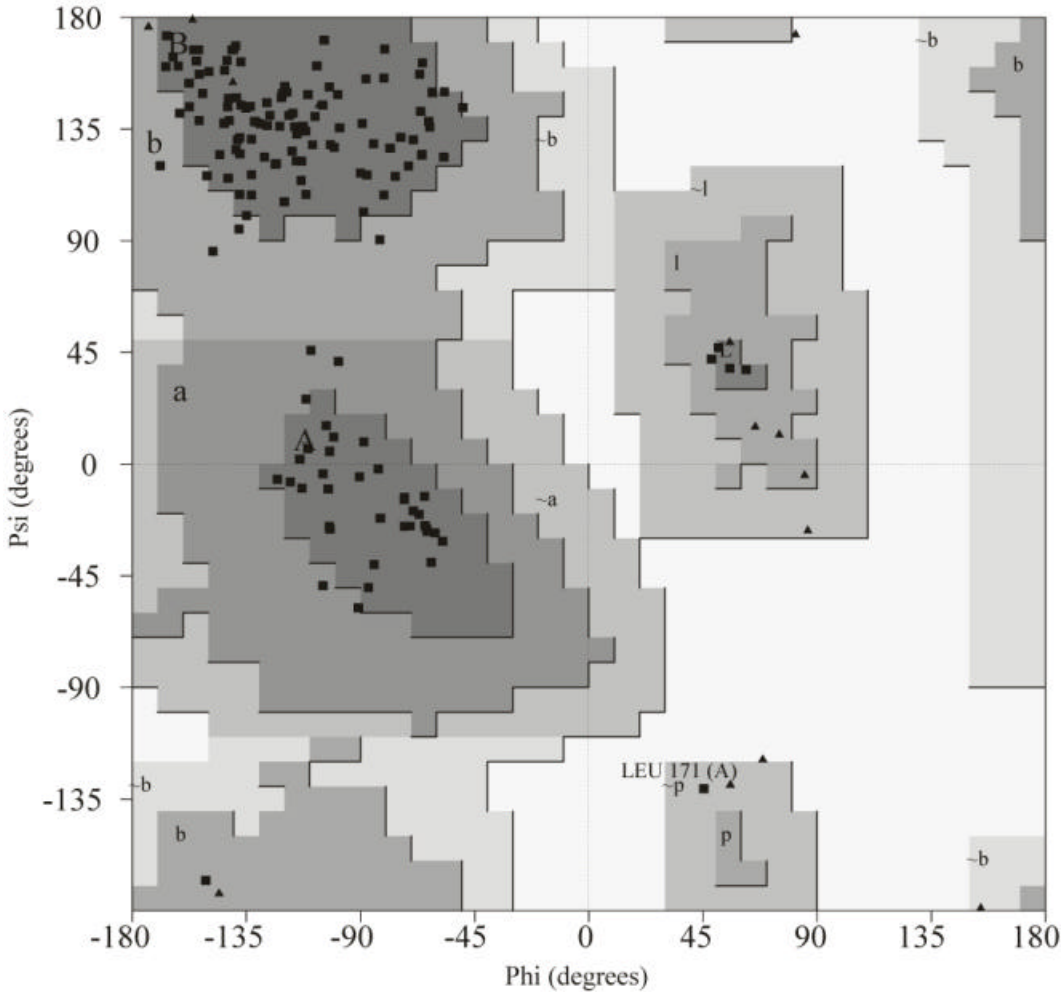
The initial crystal structure of the amino-terminal LG domain of SHBG has been solved in complex with DHT with the multiple isomorphous replacement method (M.I.R.). The structure was refined to a crystallographic R-factor of 20.5 % (R_{Free}=25.1 %) for all reflections between 40 and 1.55 Å resolution. An overview of the refinement statistics can be found in **Table 3.1**. 90 % of all residues are located in the most favorable regions of the Ramachandran plot and no residues are in disallowed regions (**Figure 3.2**) (Laskowski *et al.*, 1996). Most of the residues have an excellent electron density expected at this high resolution. The final model consists of 170 residues and 123 solvent molecules. Beyond Cys188 no density was observed for the remaining 17 residues, which include the linker region connecting the amino- and carboxy-terminal LG domains in the complete SHBG monomer. Likewise, no density was observed for the 12 amino-terminal residues. The structure contains a DHT molecule and a calcium ion. Both are clearly visible in the electron density map. Intriguing local disorder prevents the localisation of the six residues from Pro130 to Arg135. A strong difference density peak is observed in the vicinity of this loop region. I later observed that this strong density peak originated from an additional metal ion

bound in proximity of the loop (sections **3.2** and **4.3**). Although poorly conserved, residues within this loop region, such as Lys134 and Arg135, have been observed to contact the steroid ligands as described in the introduction (section **1.2.3)** of this thesis. The loop would cover the steroid binding site and possibly regulates access of ligands to the steroid-binding pocket.

Table 3.1 Refinement statistics.

Resolution (Å)	40-1.55
No. protein residues	170
(residues missing in the model:	1 - 12, 130 - 135, 189 - 205)
No. solvent molecules	123
No. reflections working / free	22208 / 2461
R / R_{FREE} (%)	20.6 / 24.9
r.m.s. deviations [a]	
bond length (Å)	0.013
bond angles (Å)	0.030
r.m.s. deviations B-factors	
bonded atoms main chain ($Å^2$)	2.79
bonded atoms side chains ($Å^2$)	3.58

[a] root mean square deviations given from ideal values



Plot statistics		
Residues in most favored regions (A, B, L)	126	90.0 %
Residues in additional allowed regions (a, b, l, p)	13	9.3 %
Residues in generously allowed regions (-a, -b, -l, -p)	1	0.7 %
Residues in disallowed regions	0	0.0 %
Number of non-glycine and non-proline residues	140	100.0 %
Number of end-residues	2	
Number of glycine residues	15	
Number of proline residues	13	
Total number of residues	170	

Figure 3.2 Ramachandran plot.
Non-glycine residues are depicted as filled squares; filled triangles represent glycines.

3.1.3. Overall structure of the N-terminal LG-domain of SHBG

The domain has a spherical shape with a diameter of about 40 Å and consists of two seven-stranded, antiparallel β-sheets packed on top of each other (**Figure 3.3**). Eight of these strands, namely β-strands 1 to 4 and 11 to 14 form a jellyroll motif (Stirk *et al*., 1992), whereas β-strands from 5 to 7 and from 8 to 10 connect to their immediate neighbours following up and down topology. Only two single-turn α-helical segments are present. They are adjacent in space and connect strands from 2 to 3 and from 12 to 13 within the jellyroll motif. A 3_{10} helical turn is present in the 15 residue-long insertion that connects β-strands 11 and 12. Only one connection, namely the inter-sheet crossing between strands 10 and 11, could not be located in the electron density. A highly conserved disulphide bridge present in all LG domains is formed between Cys164 from strand 12 and the carboxy-terminal residue Cys188 from strand 14. Calcium is coordinated to residues from two loop segments, namely to the carboxylate group of the side chain of Asp50 and the carbonyl oxygens of Glu52 from the loop connecting β-strands 3 and 4, and Ala160 from the loop between β-strands 11 and 12, as well as to three solvent molecules.

3.1.4. The laminin G-like domain fold

Searching the protein data bank (Berman *et al*., 2000) reveals that the structure of the amino-terminal LG domain of SHBG is closely related to the pentraxin serum amyloid P component (SAP) (Emsley *et al*., 1994). Among all Cα positions in both structures, 150 can be superimposed with a r.m.s.d. of 3.0 Å. This relatively high deviation mainly results from small differences in the overall orientation of the two β-sheets in the β-sandwich. Both proteins share the jellyroll fold common to a number of functionally diverse proteins, such as neuraminidases, glucanases and lectins (Stirk *et al*., 1992).

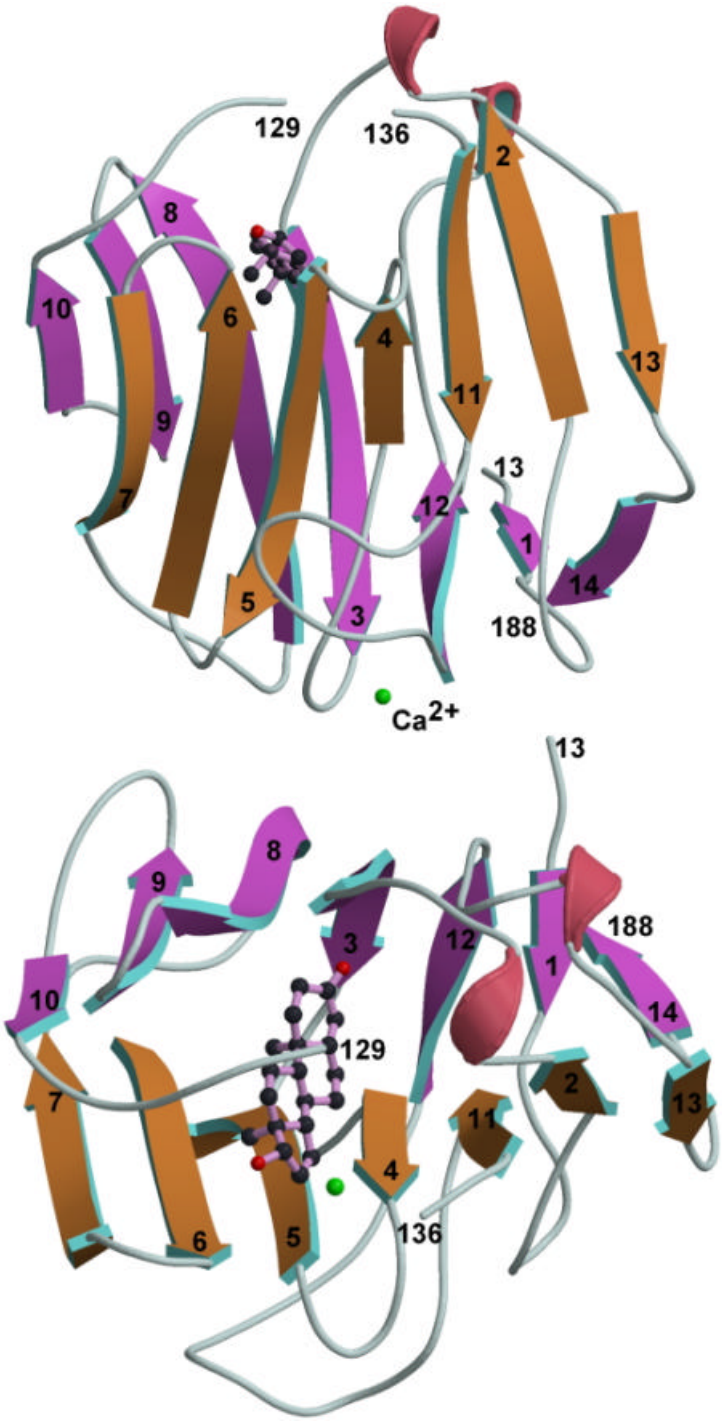


Figure 3.3 The LG domain fold in SHBG.
a) and b) Ribbon representations of the N-terminal domain of SHBG in two orthogonal orientations. The β-strands of the two β-sheets forming a β-sandwich are colored in purple and orange. The steroid 5α-DHT is shown in a ball-and-stick representation. The segment Pro130-Arg135 is disordered and not visible in the electron density. The calcium ion is shown as a green dot (figure generated with MOLSCRIPT and RASTER 3D (Kraulis, 1991; Merritt, 1994)).

The fact that LG domains are highly related to SAP was recently revealed by the determination of the crystal structure of the ectodomain of neurexin-1β, LG4 and LG5 domains of laminin a2 chain (Hohenester *et al*., 1999; Rudenko *et al*., 1999; Tisi *et al*., 2000). This corroborated previous predictions based on consensus sequence string searches, which related both the LG domain and the unresolved thrombospondin N fold to pentraxin (Beckmann *et al*., 1998). However, there are several significant differences between SHBG and SAP.

The amino-terminal G domain of SHBG, as well as the ectodomain of neurexin-1β, lacks the long α-helix connecting β-strands 12 and 13 in SAP. Furthermore, the location of both steroid- and calcium-binding sites in SHBG differ significantly from the location of the sugar- and calcium-binding sites in SAP and lectins (Emsley *et al*., 1994). In SHBG, these binding sites are located opposite each other on the rim of the β-sheet sandwich, whereas the sugar- and calcium-binding sites in SAP and the lectins are located on identical faces of the same β-sheet. Finally, different regions on the surface mediate oligomer formation in SHBG, SAP and lectins, such as concanavalin A (Hardman *et al*., 1972).

3.1.5. Dimerization of SHBG

In blood, SHBG exists as a homodimer (Westphal, 1986) and the analysis of the crystal structure of SHBG indicates that homodimerization occurs through a two-fold symmetry axis located at the edge of the β-sheet sandwich of each SHBG monomer (**Figure 3.4**). This symmetry axis places strand β7 of one monomer next to strand β10 of the other monomer and vice versa. As a consequence, the main-chain hydrogen bond pairing characteristic for β-sheets extends across the interface, and two contiguous 14-stranded β-sheets are generated in the SHBG homodimer. In total 8 main chain : main chain hydrogen bonds are formed across the interface, and 24 residues from each monomer participate in the dimerization. Each monomer contributes about 760 $Å^2$ to the interface surface. Contributions are from four different segments of SHBG, i.e., from the loop connecting strand β5 to β6; from strand β7; from strand β10, and from the large insertion between strand β11 and β12 (**Figure 3.5**). However, strand β7 and β10 account for about 580 $Å^2$ (76 %) of the interface surface, and far

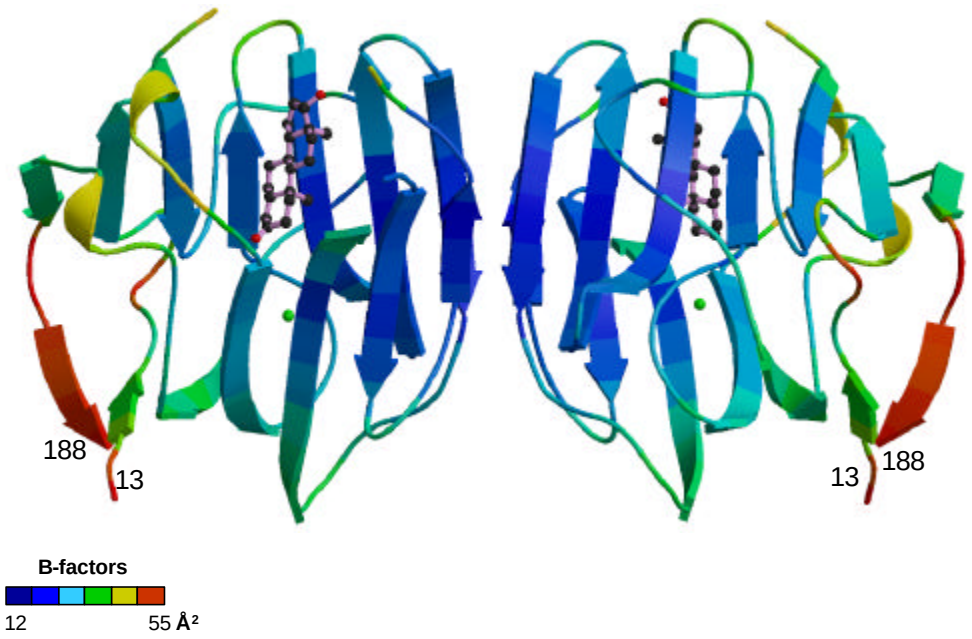
exceed the contributions of the other segments. The central region of the interface is very hydrophobic in nature and involves residues Ala85, Leu87, Val89, Leu122 and Leu124, which are strictly conserved in SHBGs from different species, as well as the largely conserved residues Ala91 and Val121 (18). The hydrophobic core is surrounded by inter-β-sheet main chain : main chain hydrogen bonds, as well as a number of hydrophilic side chain contacts provided by residues Arg75, Glu120, Arg125, Gln126 and Arg154 (**Figure 3.4(b)**). The side-chains of each of these residues contribute more than 40 $Å^2$ to the interface.

Five different observations support the proposed dimer as a biologically relevant entity. First, the interface area is as large as expected for a permanent dimer with respect to the size of the protein (Jones and Thornton, 1996). Second, ultracentrifugation experiments showed that SHBG is dimeric under the crystallization conditions (**Figure 3.6**). Third, the identical dimer was observed in the tetragonal crystal form of SHBG while all other packing contacts differ between the two crystal forms. Fourth, the distribution of atomic displacement factors supports the proposed dimerization model. The expected picture with the lowest displacement factors (thermal mobility) in the core of the protein, and higher displacement factors at the surface of the protein, is only obtained when considering the proposed dimer as the appropriate conformation (**Figure 3.4(a)**).

Finally, to support this model (Avvakumov *et al.*, 2001) experiments were performed to abolish the dimerization using the full length glycosylated protein. This was achieved by introducing larger side-chains, which would cause severe steric clashes at the proposed dimerization interface. The substitutions V89E and L122E have an increased electrophoretic mobility and chromatographic profile consistent with the presence of only a monomer.

Thus, disruption of the hydrophobic patch between strands β7 and β10 completely abolishes dimerization, the hydrophobic side-chains pack tightly within the dimer interface and the interface shape complementarity is high.

a)



b)

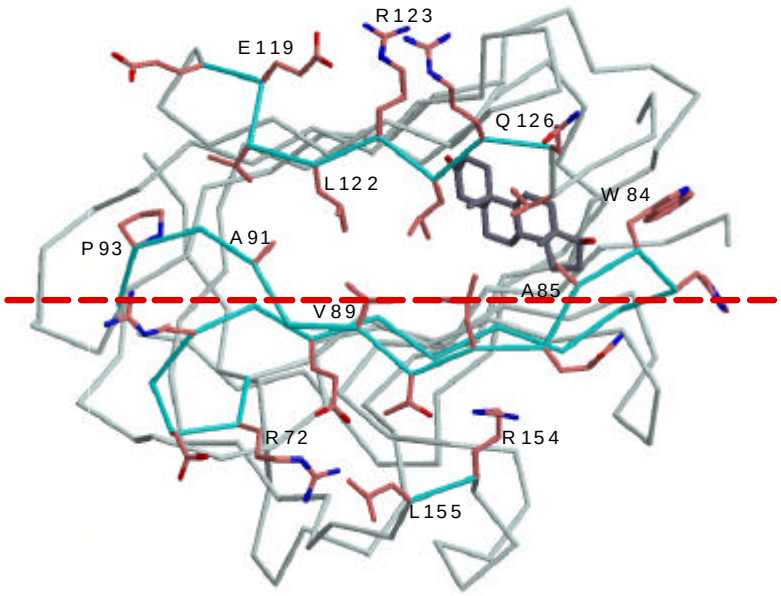


Figure 3.4 Dimerization of SHBG.
(a) Ribbon representation of the SHBG dimer colored according to the atomic displacement factors (temperature factors). (b) Detailed view of the homodimer interface. For clarity, only one monomer is displayed. The side chains of all residues that participate in the interface are shown. A dashed line indicates a 2-fold symmetry axis across the dimer interface between monomers (figures generated with MOLSCRIPT and RASTER 3D (Kraulis, 1991; Merritt, 1994)).

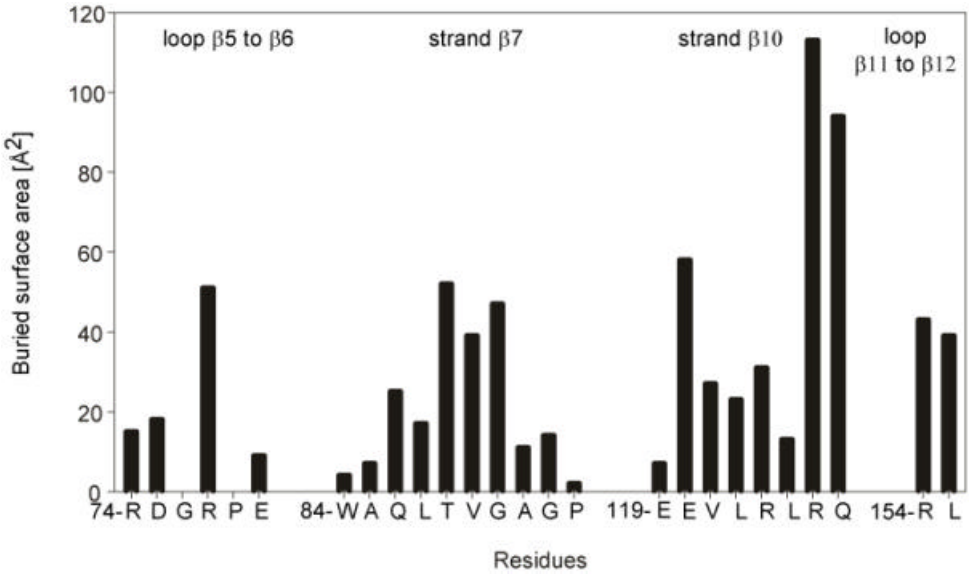


Figure 3.5 Surface area contributed by individual amino acids to the dimer interface.
The values were calculated from differences in the solvent accessibility of individual amino acids in the context of the monomer and the dimer by using program areaimol (CCP4, 1994). As indicated in the upper part of the graph, residues from four different segments of SHBG contribute to the interface.

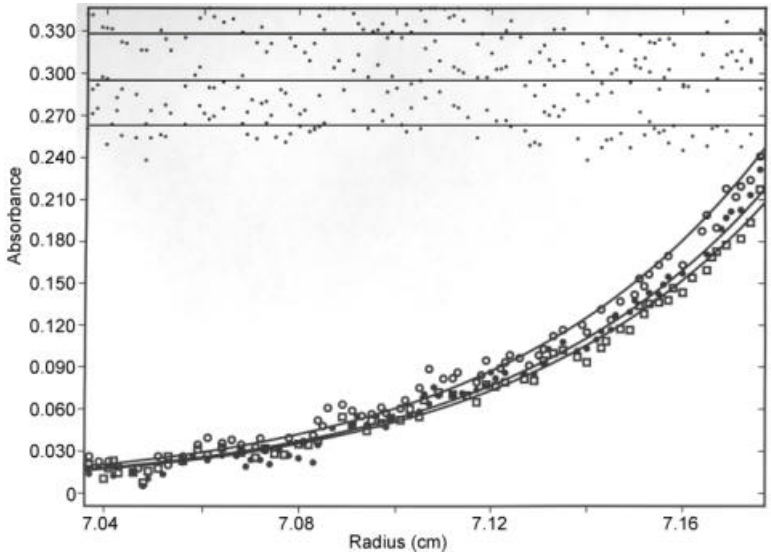


Figure 3.6 Radial absorbance distributions of SHBG (loading concentration: 0.2 mg/ml) at sedimentation equilibrium.
The data recorded at 275 nm (open circles), 280 nm (filled circles) and 285 nm (squares) were fitted simultaneously using the program POLYMOLE (Behlke *et al*., 1997), resulting in a molecular mass of 45.1±1.1 kDa. Residuals (above) are given in twofold amplification.

3.1.6. The steroid binding site

The steroid ligand 5α-DHT (**Figure 3.7**) intercalates into the hydrophobic core of the amino-terminal LG domain of SHBG in between the two β-sheets (**Figure 3.8**). Its oxygen atom at atom position C3 of ring A points into the interior of the protein and is anchored to Ser42, which is strictly conserved among species. The steroid atom numbering follows the established nomenclature and is shown in the top left side of **Figure 3.7**. The steroid-binding pocket is lined by residues contributed from eight β-strands, and is predominantly hydrophobic. The main binding contributions are from Phe67, Met107, Met139 (contact surface > 20 $Å^2$). Two additional hydrophilic contacts are formed between the hydroxyl group at C17 and the strictly conserved residues Asp65 and Asn82. The conservation of Gly58 also appears to be important because any side chain at this position would generate steric clashes with atoms C6 and C7 of the steroid. The conformation of 5α-DHT does not change when bound to SHBG. Its 19 carbon and 2 oxygen atoms can be superimposed on the crystal structure of unbound 5α-DHT with an r.m.s.d. of 0.11 Å. The structure of the human SHBG steroid-binding pocket is in agreement with mutagenesis data (Sui *et al*., 1992; Bocchinfuso and Hammond, 1994) and photolabelling experiments (Grenot *et al*., 1992; Kassab *et al*., 1998). The initial crystal structure of SHBG displays disorder for the loop segment covering the steroid-binding site between the residues Pro130 and His136. Lately, the conformation of this region has been shown by solving the crystal structure of EDTA-soaked crystals and will be described in the next section of thesis. Of the residues from the loop segment, only Lys134 is in direct contact with the steroid. Its side chain makes a hydrogen bond with the 17β-hydroxy group of DHT. The side-chain of Leu131 makes a hydrophobic contribution to the steroid surrounding.

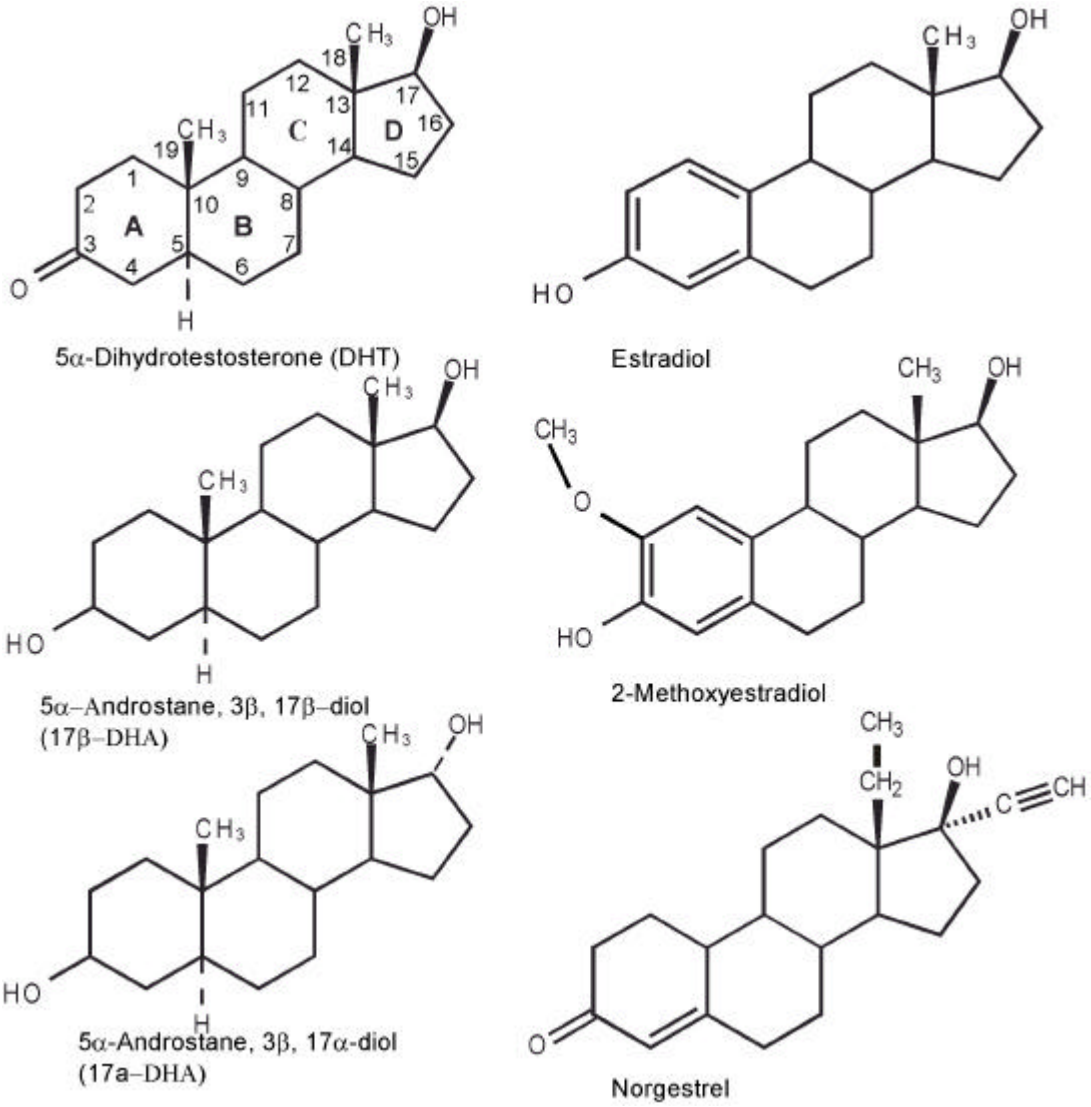


Figure 3.7 The chemical structures of various SHBG ligands.
The standard atom numbering for steroids is shown for DHT (top left).

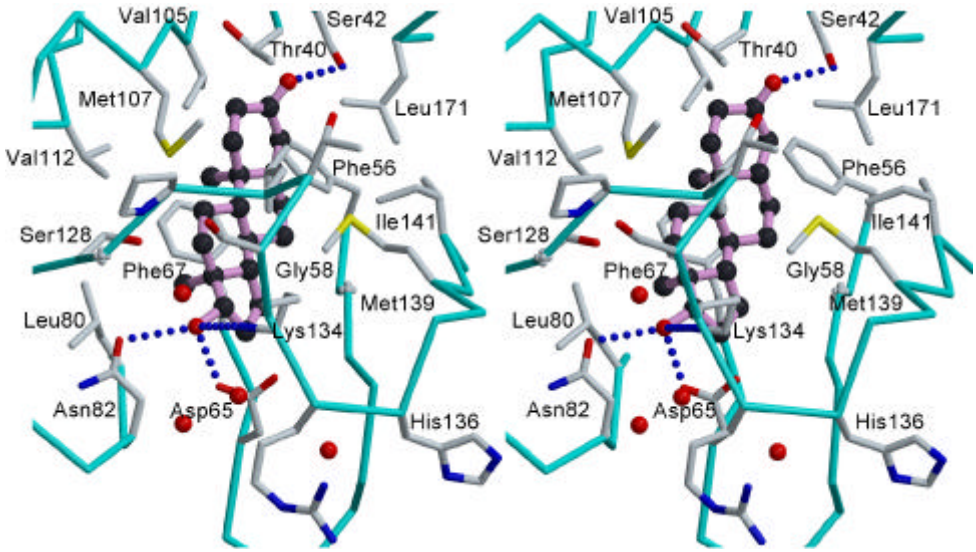


Figure 3.8 Stereo representation of the steroid-binding pocket of SHBG in complex with DHT.
The conformation of the loop region between the residues Pro130 and Arg135 covering the steroid-binding pocket is shown (EDTA-soaked crystal structure, section **3.2**). All side chains that are in contact with the steroid are displayed. Water molecules are shown in red balls (figure generated with MOLSCRIPT and RASTER 3D (Kraulis, 1991; Merritt, 1994)).

3.2. The crystal structure of SHBG including the disordered loop region

3.2.1. The crystal structure of SHBG after soaking the crystals with EDTA

An additional metal ion is observed in the original crystal structure bound in close proximity to the steroid-binding pocket. To determine the crystal structure in the absence of any contaminations from divalent ions the trigonal crystals were soaked with EDTA.
The crystal structure of the EDTA-soaked trigonal crystals was determined at 1.7 Å and refined to a crystallographic R-factor of 19.4 % (free R-factor 24.5 %; **Table 3.2**). In this structure 85 % of all residues are located in the most favorable regions of the Ramachandran plot and no residues in disallowed regions. This structure compares to the original crystal structure with a r.m.s. deviation of 0.365 Å for all main-chain atoms of equivalent residues.
The interesting feature of this structure is that the loop segment between Pro130 and Arg135 covering the steroid-binding pocket is clearly visible in the electron density map. No changes in the conformation of the main-chain or in the orientation of the side-chains occur in the calcium-binding site. A water molecule replaces calcium and as expected for hydrogen bonds, the distances to the protein functional groups are slightly increased (**Figure 3.10**).

3.2.2. The crystal structure of SHBG in a tetragonal crystal form

The crystal structure of a tetragonal crystal form of the N-terminal LG domain of SHBG has been solved at a resolution of 2.35 Å by molecular replacement using the previously described crystal structure of the complex between SHBG and 5α-dihydrotestosterone ("original" structure) as a search model. The structure was refined to a crystallographic R-factor of 19.3 % (free R-factor = 26.4 %) with low deviations from ideal model geometry (**Table 3.2**). A total of 72 solvent molecules were identified compared to the 123 water molecules observed in the original structure. Of all residues, 88 % are located in the most favorable regions of the Ramachandran plot and one residue is observed in disallowed region (**Figure 3.9**) (Laskowski *et al.*, 1993). The r.m.s deviation between the main-chain atoms of the tetragonal crystal form and the original trigonal crystal form is as large as 0.96 Å.

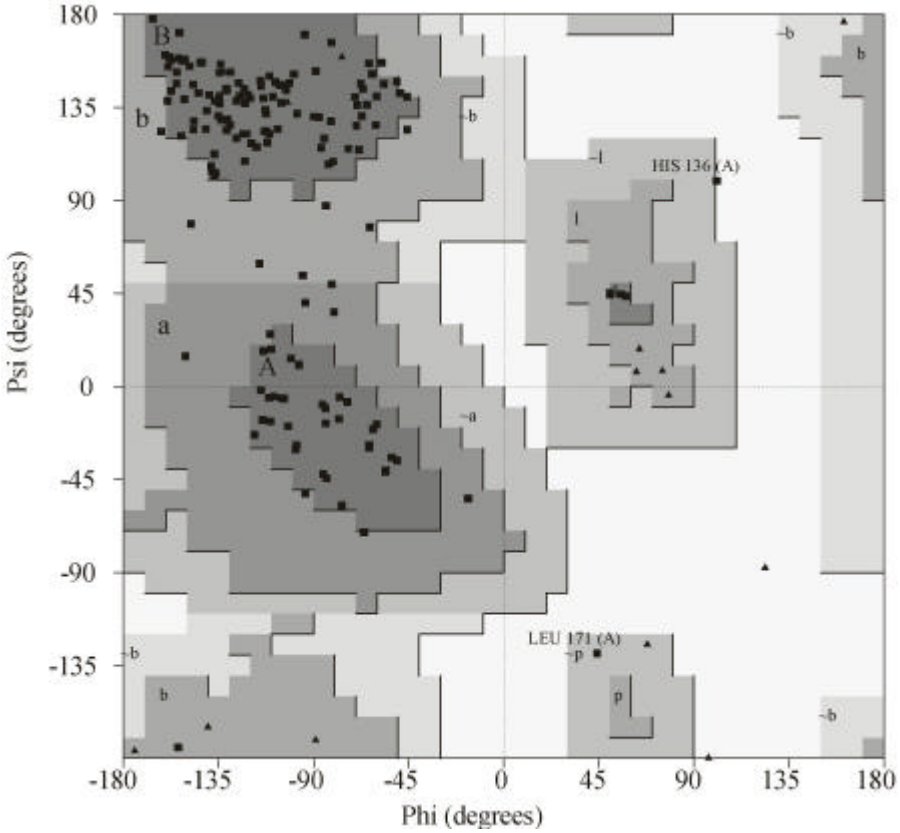
This value drops to 0.32 Å when two segments consisting of residues 21 to 24 and 180 to 185 located near the N- and C-termini of SHBG are omitted from the superposition. These two segments are juxtaposed in space and the conformation of these segments is influenced by differences in the crystal packing contacts between the two crystal forms.

In contrast to the trigonal crystal form, in the tetragonal crystal form, as well as in the EDTA soaked trigonal crystals described above (section **3.2.1**), the previously disordered loop segment 130 to 135 becomes ordered and no hint for a metal ion bound in close proximity of the steroid can be found. Whereas in case of the EDTA soaked crystals it is obvious that any divalent metal ions are removed, two possible explanations exists for the absence of such ions in the tetragonal crystals. In contrast to the original crystal structure, the crystallized SHBG variant contains a polyhistidine tag and was purified over a nickel agarose column. In order to circumvent aggregation, the protein was crystallized in high imidazol concentrations and both these manipulations potentially remove any contaminations with divalent metal ions from the protein production.

Table 3.2 Refinement statistics.

	Trigonal crystals EDTA-soaked	Tetragonal crystal
Resolution (Å)	20-1.7	40-2.35
Number of residues	177	177
Number of water molecules	89	72
R-value (%)	19.4	19.3
R_{free}-value (%)	24.5	26.4
Average B-factor (Å^2)	36.2	35.6
r.m.s.deviation [b]		
bond lengths (Å)	0.012	0.010
bond angles (Å)	0.037	0.039

[b] root mean square deviation given from ideal values



Plot statistics		
Residues in most favored regions (A, B, L)	128	87.7 %
Residues in additional allowed regions (a, b, l, p)	16	11.0 %
Residues in generously allowed regions (-a, -b, -l, -p)	1	0.7 %
Residues in disallowed regions	1	0.7 %
Number of non-glycine and non-proline residues	146	100.0 %
Number of end-residues	2	
Number of glycine residues	15	
Number of proline residues	14	
Total number of residues	177	

Figure 3.9 Ramachandran plot of the crystal structure of SHBG as observed in the tetragonal crystal form.
Non-glycine residues are depicted as filled squares; filled triangles represent glycines.

3.2.3 The structure of the loop segment covering the steroid-binding pocket

The segment between the residues 130 and 136 in the loop connecting β-strands 10 and 11 is well ordered in the structure of SHBG in the tetragonal crystal form and the EDTA-soaked crystals (**Figures 3.11, 3.12**). Loop covers the steroid-binding pocket. In this loop region, residues 130 to 131 are in an extended conformation. Residues 131 to 134 form a single 3_{10} helical turn with a hydrogen bond between the main-chain carbonyl oxygen of Leu131 and the main-chain nitrogen amino-group of Lys134. The following residues (Lys134 and Arg135) adopt a more extended conformation. Of the residues from the loop segment, only Lys134 is in direct contact with the steroid. Its side chain makes a hydrogen bond with the 17β-hydroxy group of DHT. In the EDTA-soaked crystals, the distance between the Lys134-Nε and atom O17 of the steroid is 3.54 Å. This is in agreement with affinity labeling experiments. Residue Lys134 has identified as a part of the steroid-binding pocket in binding tests with 17β-bromoacetoxy-DHT (Namkung *et al*., 1990). Although the side-chain of Leu131 is not in direct van der Waals contact with the steroid, the short distance of 4.6 Å between Leu131-Cδ2 and atom C12 of the steroid reflects its close proximity. Its side-chain points into a hydrophobic pocket formed by residues Leu34, Met107, Leu131, Met139 and Leu171.

On average, the thermal displacement factors in the loop segment are higher when compared to the rest of the protein. The highest displacement factors are observed for the main-chain atoms of residues 133 and 134 in both crystal structures. When summarised over both residues, they are as high as 85 and 65 $Å^2$ in the EDTA-soaked trigonal crystals and in the tetragonal crystals, respectively. This is significantly higher than the observed averages for all atoms within both protein structures (**Table 3.2**). The elevated thermal displacement factors of all residues in the loop segment suggest that this region has enhanced conformational flexibility and structural elements within it can rapidly fold and unfold. This loop is exposed to the surface of the protein and presumably functions as a flap that regulates an essential access of the ligands to the steroid-binding pocket.

Thus, the crystal structures of the tetragonal crystal form and the EDTA-soaked trigonal crystals of SHBG revealed the conformation of the loop segment Pro130-Arg135.

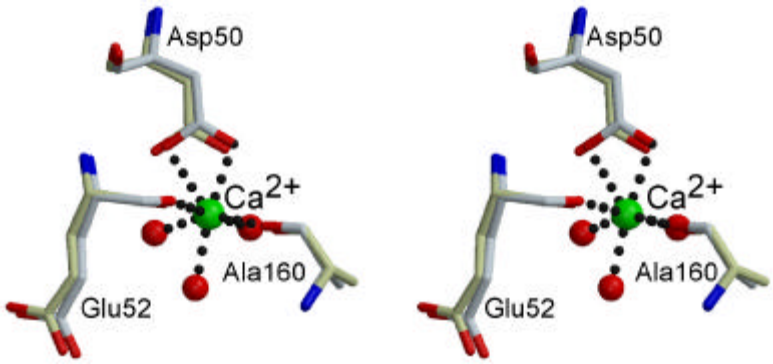


Figure 3.10 Stereo representation of the calcium-binding site of the original crystal structure SHBG superposed with the EDTA-soaked crystal structure.
All side chains of the original crystal structure participating in calcium binding are shown in gray. All side chains of the EDTA-soaked crystal structure are displayed in light yellow. The calcium ion is shown in green, water molecules are displayed in red balls (figure generated with MOLSCRIPT and RASTER 3D (Kraulis, 1991; Merritt, 1994)).

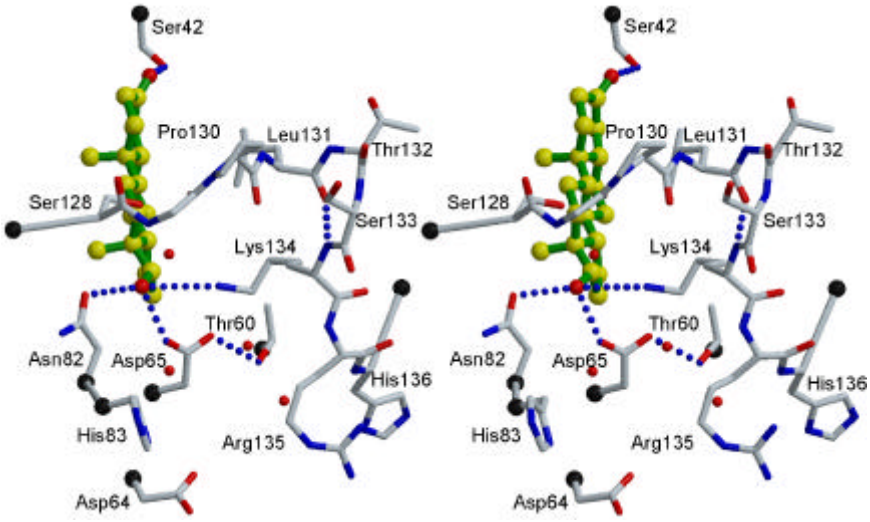


Figure 3.11 Stereo representation of the loop segment covering the steroid-binding pocket. Water molecules are shown in red balls (figure generated with MOLSCRIPT and RASTER 3D (Kraulis, 1991; Merritt, 1994)).

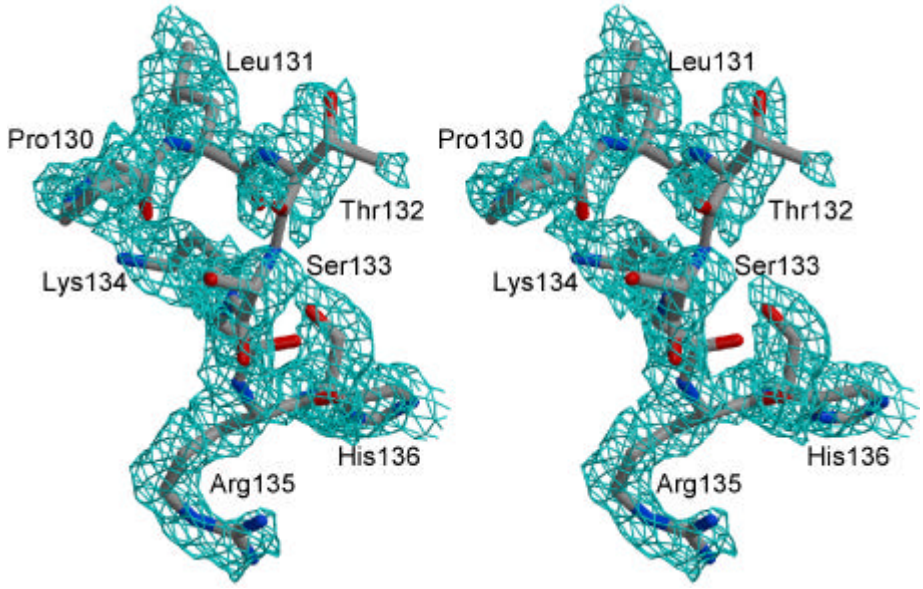


Figure 3.12 Stereo representation of the 2Fo-Fc ó$_A$-weighted electron-density map contoured at 0.8 ó showing the loop segment covering the steroid-binding pocket (figure generated with BOBSCRIPT, MOLSCRIPT and RASTER 3D (Kraulis, 1991; Merritt, 1994; Esnouf, 1997)).

3.3. Structural determinants of the steroid-binding specificity of SHBG

In order to investigate the structural determinants of the steroid-binding specificity of SHBG different SHBG-steroid complexes were crystallized and the structural features of each ligand studied in details.

3.3.1. Crystallization of different SHBG-ligand complexes

Crystals of SHBG in complex with different ligands, such as estradiol, 17β-DHA, 17α-DHA, and norgestrel were obtained by reproducing the conditions for the trigonal crystal form with some variations. Crystals of all SHBG-steroid complexes described here belonged to the space group R32 with small variations in cell dimensions (**Table 2.3**, **Figure 3.13**).

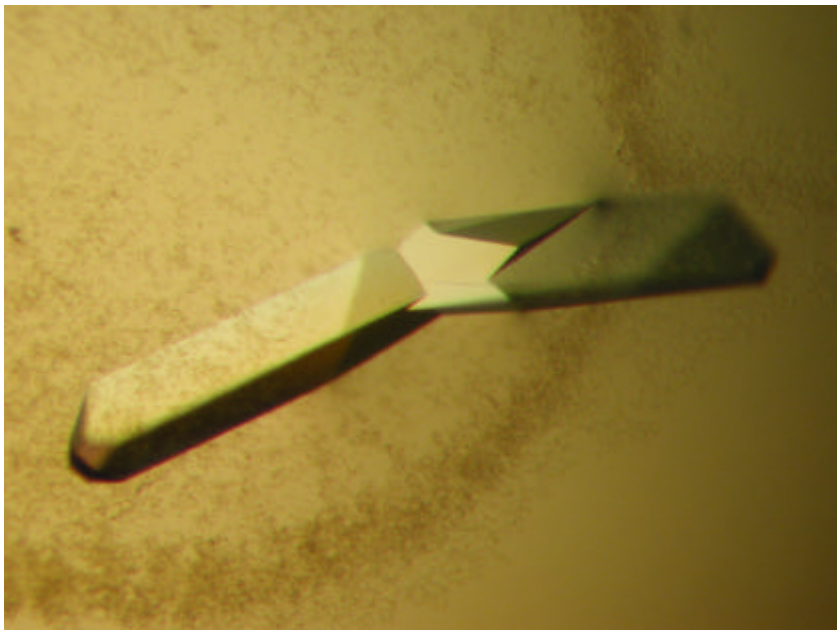


Figure 3.13 Crystals of SHBG in complex with methoxyestradiol, space group R32, dimensions 200×200×1000 μm^3.

3.3.2. SHBG in complex with estradiol

Estradiol was chosen for our structural studies because it is one of the most potent natural estrogens. Estradiol differs from DHT in that ring A is aromatic, C19 is missing and the oxo-group at atom C3 (3-oxo group) is replaced by an hydroxyl group (**Figure 3.7**).

The crystal structure of SHBG in complex with estradiol has been solved at 1.8 Å resolution. The structure was refined to a crystallographic *R*-factor of 20.4 %. (R_{free}= 23.7 %) for all reflections between 20-1.8 Å resolution (**Table 3.3**). About 88 % of all residues are located in the most favorable regions of the Ramachandran plot and no residues are in disallowed region. This structure compares to the structure of the EDTA soaked crystals with a r.m.s. deviation of 1.16 Å for all main-chain atoms of equivalent residues.

The structure shows an unexpected result: estradiol is observed to bind in a "reverse" mode. It flipped over and moved to 1.3 Å to the left of the position of DHT in the original structure (**Figure 3.14**). Thus, the hydroxyl group at atom C17 forms a hydrogen bond with Ser42, while the hydroxyl group at the C3 atom makes an hydrophilic contact with Asn82, Asp65 and Lys134. The hydroxyl group at atom C17 of estradiol replacing the 3-oxo group of DHT in the original crystal structure of SHBG donates the hydrogen bond accepted by the carbonyl oxygen of Val105. The single methyl group at atom C13 of estradiol is located on the right side of the steroid binding pocket, in the opposite orientation to the methyl groups of DHT in the previous DHT complex structure. The methyl group displaces Leu171 by about 0.98 Å when compared to the original DHT complex. This movement induces a small shift in the adjacent residues belonging to the single α-helical turn formed by residues Asp172 and 176. The displacement of the position of Thr38 (0.98 Å) takes place because of its hydrophilic side-chain is in contact with Asp172. The loop segment between the residues Pro130 and His136 is well ordered and comes close to the steroid. The positions of the Cα atoms of the residues Leu131, Thr132, Ser133 and Lys134 are shifted by about 1.25, 1.4, 1.7 and 1.6 Å respectively. Leu131 makes a Van de Waals contact with the methyl group of estradiol (distance 3.6 Å), while in the structure of the EDTA soaked crystals the short distance of 4.6 Å between Leu131 and atom C12 of the steroid highlights its close proximity.

Lys134 lies closely to the steroid ligand and is in tight hydrogen bond with the hydroxyl group at C3 atom of the steroid (Lys134 Nε–O3 distance 2.8 Å). This residue also forms a

hydrogen bond with the hydroxyl group of Ser128 (Lys 134 Nε–Ser128 distance 3.0 Å). Moreover, a conformational change of Trp84, i.e rotation of its aromatic ring by 180 degree, apparently, is induced by the shifting of the localization of Lys134. The positions of the Asp65, Asn82 and Ser42 did not change much comparing to the original structure.

The crystal structure of SHBG in complex with estradiol revealed unexpected differences in the orientation of the ligands in the steroid-binding pocket of SHBG.

Table 3.3 Refinement statistics.

	estradiol	**17a - DHA**	**norgestrel**	**methoxyestradiol**	**17b - DHA**
Resolution (Å)	20-1.8	20-2.0	20-2.0	20-1.75	20-2.0
Number of residues	175	176	173	174	176
Number of water molecules	96	88	64	97	93
Average B-factor ($Å^2$)	29.9	38.5	40.8	38.5	27.4
R-value (%)	20.4	19.8	21.5	21.0	18.7
R_{free}-value (%)	23.7	23.9	26.4	23.8	22.3
r.m.s. deviation [a]					
bond lengths(Å)	0.010	0.010	0.009	0.010	0.010
bond angles(Å)	1.58	1.6	1.57	1.52	1.55

[a]root mean square deviation given from ideal values

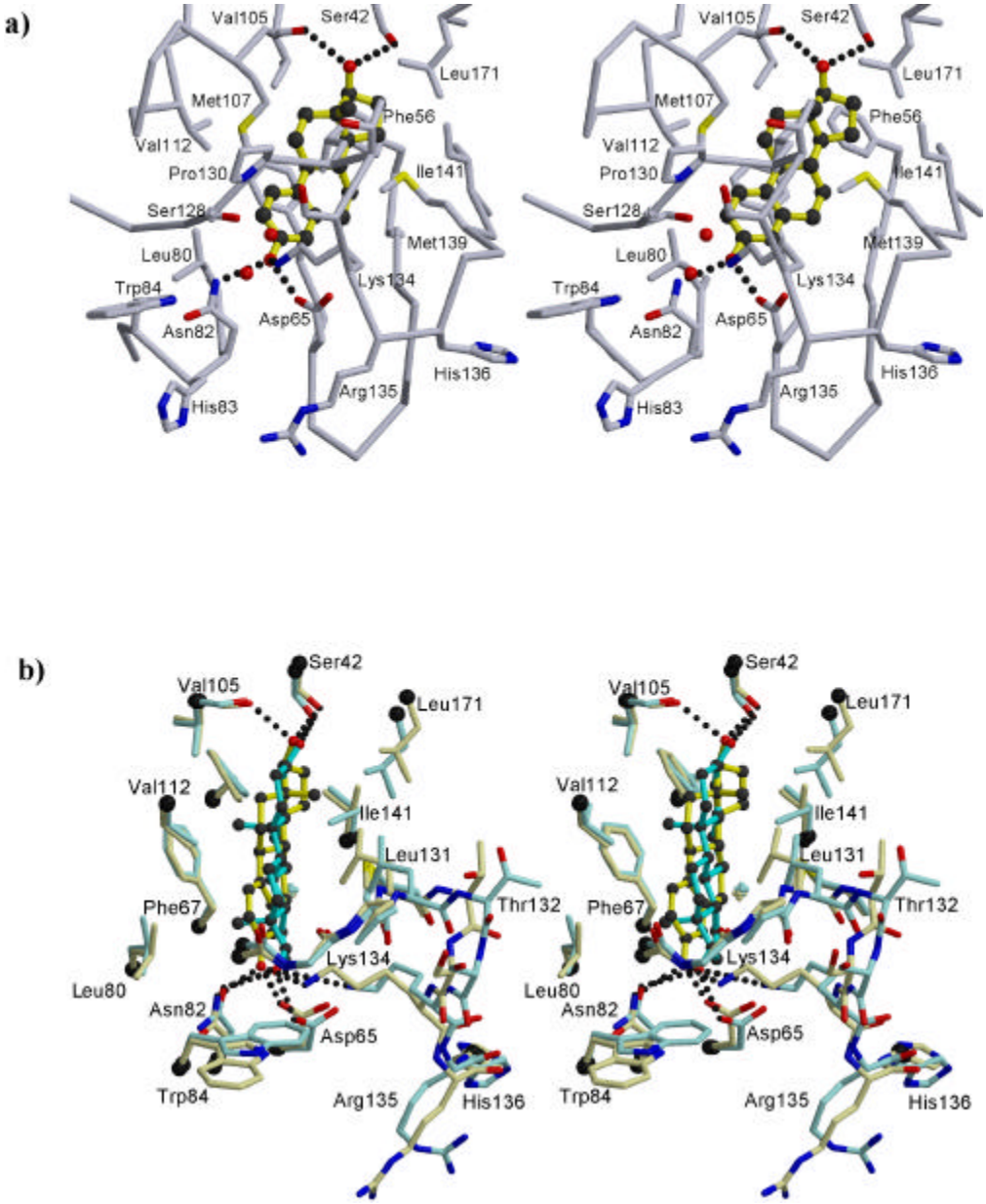


Figure 3.14 Steroid-binding pocket of the SHBG- estradiol complex.
a) Stereo representation of the steroid-binding pocket; b) comparison of the steroid-binding pockets of SHBG in complex with estradiol (yellow) and DHT (blue). All side chains belonging to the SHBG-estradiol complex are displayed in light yellow, those of the SHBG-DHT complex are shown in light blue (figure generated with MOLSCRIPT and RASTER 3D (Kraulis, 1991; Merritt, 1994)).

3.3.3. SHBG in complex with 17b - DHA

The crystal structures of SHBG in complex with DHT and estradiol showed that different steroid ligands can be bound in different orientations. To try to understand the structural features which determine the orientation of the ligands, the steroid 17β–DHA was chosen as an additional ligand for our crystallographic studies. 17β–DHA has a chemical composition inbetween DHT and estradiol. It contains two methyl groups as for DHT and two hydroxyl groups as for estradiol (**Figure 3.7**).

The crystal structure of SHBG in complex with 17β–DHA has been solved at 2.0 Å resolution. The structure was refined to a crystallographic *R*-factor of 19.3 %. (R_{free}= 23.1 %) for all reflections between 20-2.0 Å resolution (**Table3.3**). About 90 % of all residues are located in the most favorable regions of the Ramachandran plot and no residues are in disallowed regions. This structure compares to the structure of the EDTA soaked crystals with a r.m.s. deviation of 0.42 Å for all main-chain atoms of equivalent residues.

The ligand is oriented in the steroid-binding pocket in the same orientation as observed for DHT. The hydroxyl group at atom C3 points towards Ser42 and the hydroxyl group at atom C17 is hydrogen bonded to Asn82 and Asp65. Apparently, the second methyl group, which is absent in estradiol, is one of the determinants for the orientation of the steroids in the binding pocket (**Figure 3.15**). The replacement of the carbonyl group of DHT at position C3 with a hydroxy-group does not induce a change in the orientation of the bound steroid. The hydroxy-group at position C3 of 17β–DHA interacts with SHBG in a very similar way then the hydroxy-group of estradiol. As for estradiol, the carbonyl oxygen of Val105 is involved in a hydrophilic contact with the hydroxyl group at atom C3. In addition, a peptide flip occurs between Lys39 and Thr40 and an additional hydrogen bond is formed between the hydroxyl group of atom C3 and the carbonyl oxygen of Thr40.

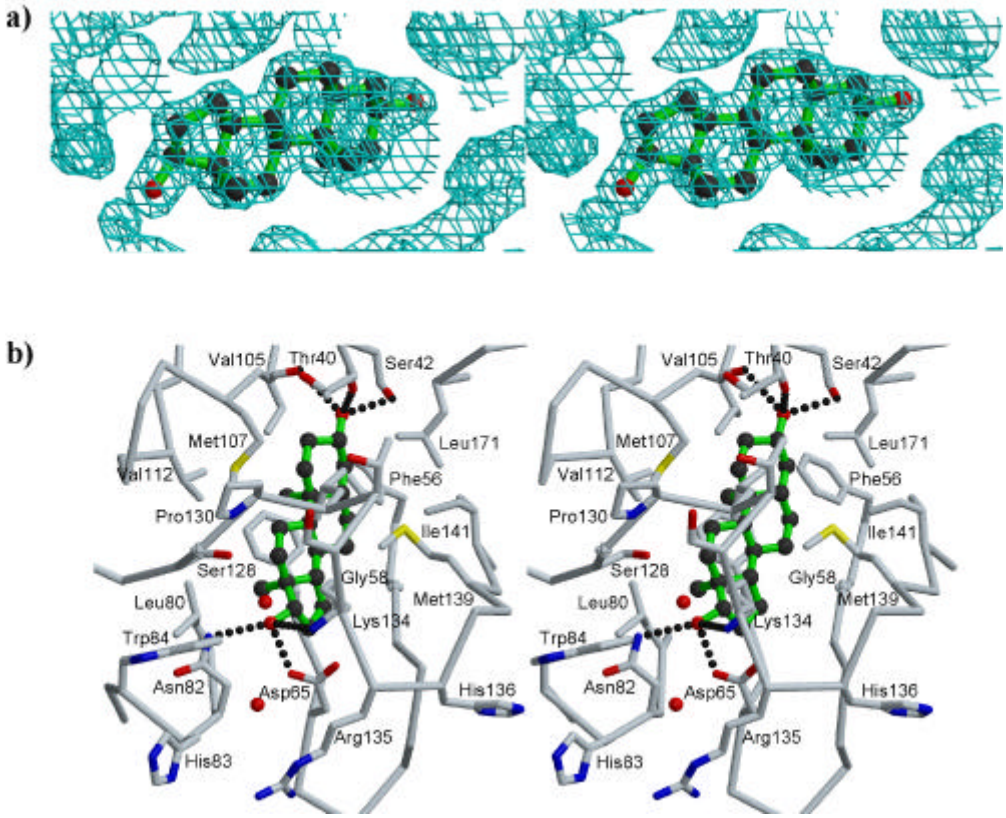


Figure 3.15 Steroid-binding pocket of the SHBG- 17β-DHA complex.
a) A stereo view of the 2Fo-Fc ó$_A$-weighted electron-density map contoured at 1.2 ó showing the steroid; b) stereo representation of the steroid-binding pocket of SHBG in complex with 17β-DHA. All side chains in contact with the steroid are displayed (figure generated with BOBSCRIPT, MOLSCRIPT and RASTER 3D (Kraulis, 1991; Merritt, 1994; Esnouf, 1997)).

The loop segment between Pro130 and Arg135 is well ordered in the crystal structure of SHBG in complex with 17β-DHA and packs closely against the steroid when compared to the structure of the EDTA soaked crystals. The shifts in the positions of the Cα atoms of Leu131, Thr132, Ser133, Lys134 corresponds to 0.66, 1.17, 1.02 and 0.54 Å respectively. Leu131 makes a hydrophobic contribution, while Lys134 creates a hydrogen bond with the hydroxyl group at atom C17 of the steroid (Lys134 Nε–O17 distance 3.8 Å).

3.3.4. SHBG in complex with 17a - DHA

The binding of 17β-steroids to SHBG was extensively studied, but there was no data on binding of 17α-ligands. To show the possibility of such binding, the ligand 17α–DHA was chosen for our structural investigations of the steroid-binding pocket of SHBG (**Figure 3.7**). The crystal structure of SHBG in complex with 17α–DHA has been solved at 2.0 Å resolution. The structure was refined to a crystallographic *R*-factor of 19.8 %. (R_{free}= 23.9 %) for all reflections between 20-2.0 Å resolution (**Table 3.3**). About 86 % of all residues are located in the most favorable regions of the Ramachandran plot and no residues are in disallowed region. This structure compares to the structure of the EDTA soaked crystals with a r.m.s. deviation of 0.42 Å for all main-chain atoms of equivalent residues.

The ligand is oriented in the steroid-binding pocket in the same orientation as observed for DHT. As in the previously described structure of SHBG in complex with 17β-DHA a peptide flip occurs between Lys39 and Thr40 and an additional hydrogen bond is formed between the hydroxyl group at atom C3 and the carbonyl oxygen of Thr40 (**Figure 3.16**). The carbonyl oxygen of Val105 is involved in an additional hydrophilic contact with the hydroxyl group of atom C3 of the steroid. The zinc binding site (section **4.3**) is partially occupied as observed in the original structure of SHBG in complex with DHT. Zinc is coordinated by two nitrogen atoms provided by the side chains of His83 and His136 and an oxygen atom contributed by the carboxylate group of Asp65. The second oxygen of the carboxylate group of Asp65 makes a hydrophilic contact with the hydroxyl group of atom C17 of the steroid. Apparently, as the result of the orientation of the His136 towards the metal ion we did not see Arg135 in this structure.

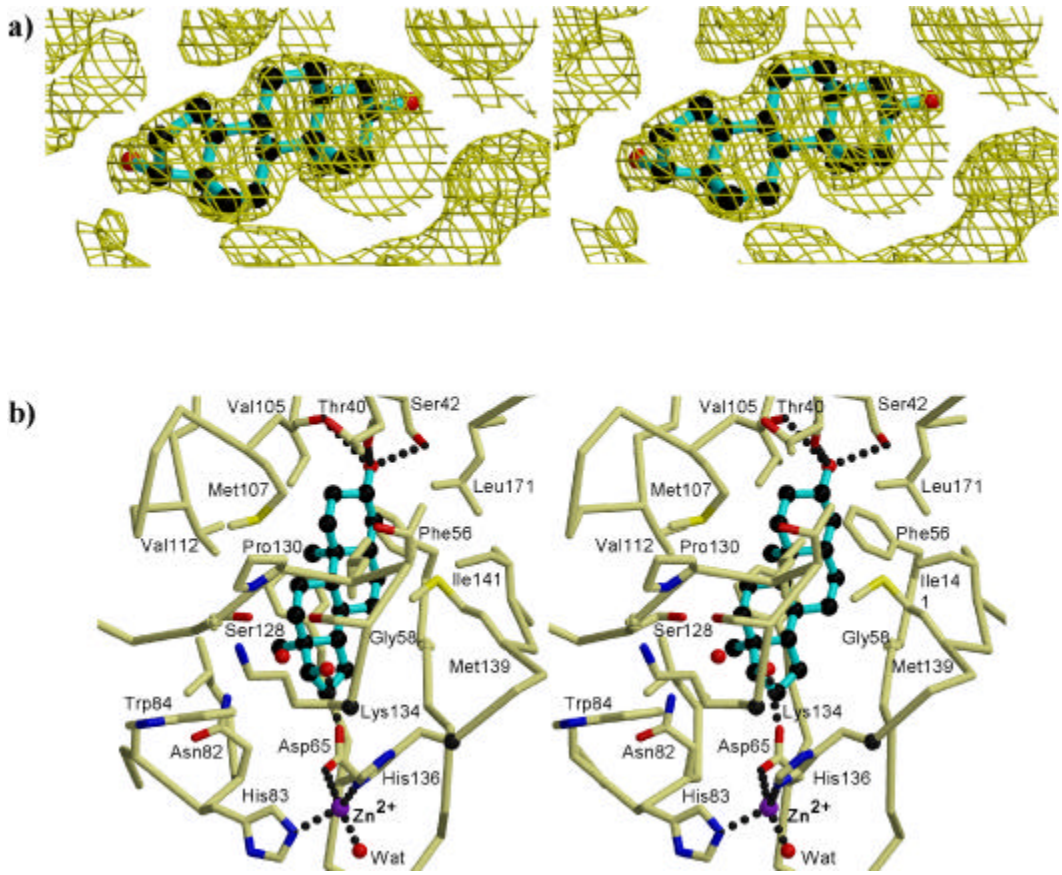


Figure 3.16 Steroid-binding pocket of the SHBG- 17α-DHA complex.
a) A stereo view of the 2Fo-Fc ó$_A$-weighted electron-density map contoured at 1.2 ó showing the steroid; b) stereo representation of the steroid-binding pocket of SHBG in complex with 17α-DHA. All side chains in contact with the steroid are displayed (figure generated with BOBSCRIPT, MOLSCRIPT and RASTER 3D (Kraulis, 1991; Merritt, 1994; Esnouf, 1997)).

The change from the β-positioning of the hydroxyl group at atom C17 to the α-positioning in 17α–DHA makes the previously observed hydrogen bond with Asn82 impossible. The same holds true for Lys134; the side chain of Lys134 adopts to the α-positioning of the hydroxyl group and points now outwards from the steroid-binding pocket. It now forms a hydrogen bond with the nitrogen atom of Pro130 (Lys134 Nε–Pro130 N distance 3.5 Å).

3.3.5. SHBG in complex with methoxyestradiol

Methoxyestradiol (**Figure 3.7**) is an endogenous metabolite of estradiol and it binds as well to SHBG. Therefore, it would be interesting to see structural changes upon this substitution at atom C2 of estradiol. Moreover, methoxyestradiol is more potent than estradiol in inhibiting receptor binding of SHBG, didn't cause an increase in intracellular AMP (Rosner *et al*., 1991; Joseph, 1994; Rosner *et al*., 1999). Perhaps, binding of methoxyestradiol different from other steroids and solving the structure with this compound could help us to explain this mechanism at a structural level.

The crystal structure of the N-terminal LG-domain of SHBG in complex with methoxyestradiol has been solved at 1.75 Å resolution. The structure was refined to a crystallographic *R*-factor of 21.0 %. (R_{free}= 23.8 %) for all reflections between 20-1.75Å resolution (**Table 3.3**). About 88 % of all residues are located in the most favorable regions of the Ramachandran plot and no residues are in disallowed region. This structure compares to the structure of the EDTA soaked crystals with a r.m.s. deviation of 1.28 Å for all main-chain atoms of equivalent residues.

The ligand is oriented in the steroid-binding pocket in the "reverse" mode, as observed for estradiol (**Figure 3.17**). The hydroxyl group at atom C17 forms a hydrogen bond with Ser42 and with the carbonyl oxygen of Val105. Intercalation of the methoxy group at position C2 of the estradiol changes the conformation of the loop segment between residues Pro130 and His136.

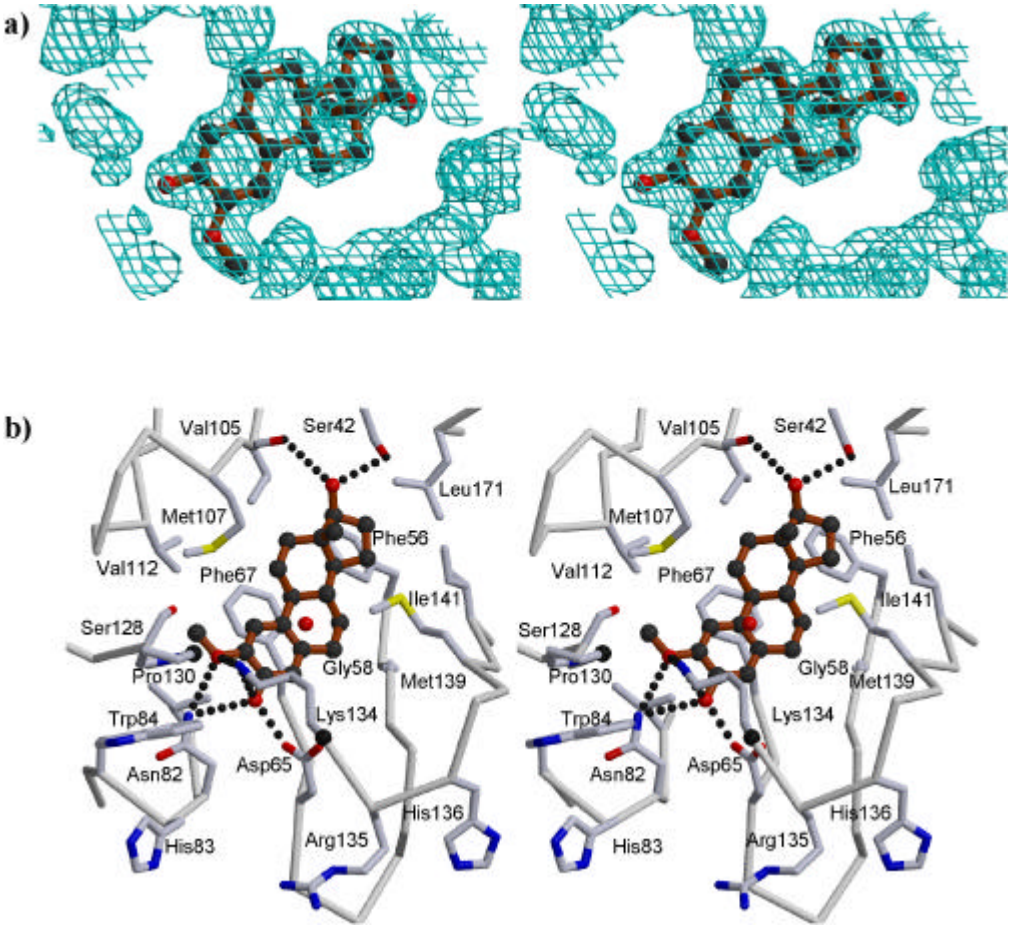


Figure 3.17 Steroid-binding pocket of the SHBG- methoxyestradiol complex.
a) A stereo view of the 2Fo-Fc ó$_A$-weighted electron-density map contoured at 1.2 ó showing the steroid; b) stereo representation of the steroid-binding pocket of SHBG in complex with methoxyestradiol. All side chains in contact with the steroid are displayed (figure generated with BOBSCRIPT, MOLSCRIPT and RASTER 3D (Kraulis, 1991; Merritt, 1994; Esnouf, 1997)).

The segment between the residues Ser128 and Lys134 is disordered (**Figure 3.17**). The part of the loop from Ser128 to Pro130 is displaced away from the steroid-binding pocket, when compared to other complexes. The displacements between the positions of the Cα atoms when comparing to the structure of the EDTA soaked crystals increases from 0.85 for Ser128 to 4.4 Å for Pro130. In contrast, residues Lys134 and Asp135 are shifted towards the steroid and are displaced by 3.7 and 3.3 Å respectively. Lys134 forms a contact with both the oxygen of the methoxygroup (distance Lys134 Nε–O2 of atom C2 of methoxyestradiol distance = 2.7 Å) and the hydroxyl group at atom C3 (distance Lys134 Nε–O3 of atom C3 of methoxyestradiol = 2.8 Å). Residue Arg135 now forms a salt bridge with residue Asp64. The nitrogen atom of Asn82 creates a hydrophilic contact with equal distances to both oxygens (Asn82 N–O3, O2 of atom C3 and C2 of methoxyestradiol distance 3 Å). Leu171 adapts to by taken the same distal position previously observed in the estadiol complex. Taking together these structural changes can't tell as anything about putative mechanism by which methoxyestradiol modulates binding of SHBG to a cell surface receptor. Further investigations such as the identification of the receptor are needed, before any conclusions about these interactions can be drawn.

3.3.6. SHBG in complex with norgestrel

Norgestrel (**Figure 3.7**) is a synthetic steroid used in medicine as an oral contraceptive. It binds to SHBG with high affinity. It is characterized by an α-ethynyl group at atom C17, an ethyl group at atom C13, a double bond between atoms C4 and C5 as for testosterone and by the absence of a C19 methyl group at atom C10.

The crystal structure of SHBG in complex with norgestrel has been solved at 2.0 Å resolution. The structure was refined to a crystallographic *R*-factor of 21.5 %. (R_{free}= 26.4 %) for all reflections between 20-2.0 Å resolution (**Table 3.3**). Only 64 solvent molecules could be located which amounts to 50 % of those located in the original crystal structure. Possibly, this is the reason for the relatively high R_{free} when compared to other SHBG structures described in this work. About 88 % of all residues are located in the most favorable regions of the Ramachandran plot and no residues are in disallowed regions. This

structure compares to the structure of the EDTA soaked crystals with a r.m.s. deviation of 0.5 Å for all main-chain atoms of equivalent residues.

The ligand is oriented in the steroid-binding pocket in the same orientation as observed for DHT. The structure is very similar to the crystal structure of the DHT complex (**Figure 3.18**). The oxygen at atom C3 of the steroid forms a hydrogen bond with Ser42 and the hydroxyl group at atom C17 creates a hydrophilic contact with Asn82 and Asp65.

In this structure the zinc binding site (section **4.3)** is partially occupied and causes disorder in the loop region between residues Leu131 and His136 as observed in the original structure. The segment between Pro130 and Leu131 adapts to accommodate the 17α-ethynyl group of the norgestrel and shifts these residues by about 1.4 Å away from their corresponding positions in the steroid-binding pocket of the DHT complex. The ethyl-group at atom C13 intercalates into the steroid-binding pocket without any rearrangements.

Thus, the structure shows how contraceptives can bind to SHBG. This might be important in the further developing of synthetic steroids for medicine, because synthetic steroids may compete with natural steroids for the SHBG steroid-binding site and, thus, alter the distribution of natural steroid hormones.

The crystal structures of SHBG in complex with different ligands revealed that the steroid-binding pocket of SHBG is very adaptable and has different possibilities to accommodate different functional groups from the ligands. The binding may occur in two different orientations, in a “forward” mode like in DHT or in the “reverse” mode like in estradiol.

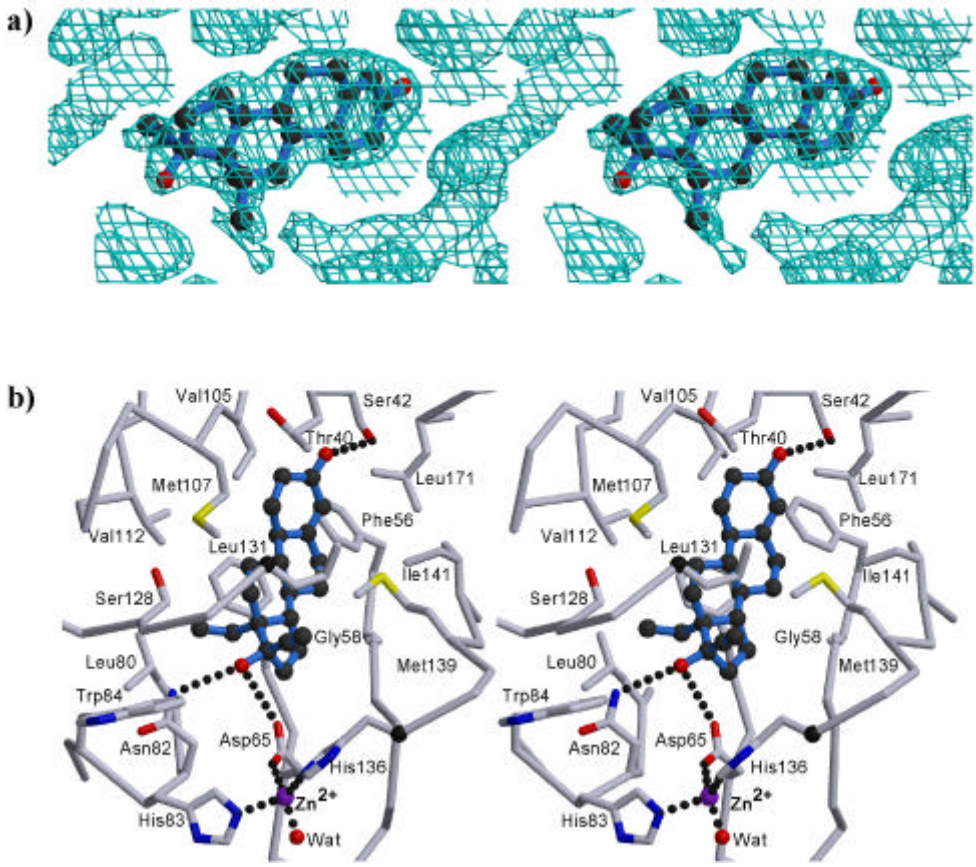


Figure 3.18 Steroid-binding pocket of the SHBG- norgestrel complex.
a) A stereo view of the composite annealed OMIT map contoured at 1.2 ó showing the steroid; b) stereo representation of the steroid-binding pocket of SHBG in complex with norgestrel. All side chains that are in contact with the steroid are displayed (figure generated with BOBSCRIPT, MOLSCRIPT and RASTER 3D (Kraulis, 1991; Merritt, 1994; Esnouf, 1997)).