4. Discussion

4.1. LG domain fold

The crystal structure of the amino-terminal LG domain of SHBG in complex with 5α-dihydrotestosterone (5α-DHT) presented here confirmed the initial prediction (Beckmann *et al*., 1998) of the LG domain structure as well as the reported recently crystal structures of the ectodomain of neurexin-1β, LG4 and LG5 domains of laminin α2 chain.(Hohenester *et al*., 1999; Rudenko *et al*., 1999; Tisi *et al*., 2000). The detailed comparison between these structures has been made in a review (Rudenko *et al*., 2001), which revealed their striking similarity. The LG domains from neurexin-1β, LG4 and LG5 domains of laminin α2 chain and SHBG have a spherical shape and consist of two seven-stranded anti-parallel β-sheets (**Figure 4.1**). The first four N-terminal β-strands and the last four C-terminal β-strands form a jelly-roll folding motif (Brändén and Tooze, 1999), whereas the remaining six-strands connect to their neighbors following up and down topology. The loop β10-β11, which undergoes conformational changes in SHBG and which is described in detail in this thesis, is well ordered in neurexin and laminin LG domain structures and apparently involved in ligand interaction. The N- and C-termini of LG-domains lie in close proximity. Unexpectedly these structures reveal close structural homology between the LG/LNS domain fold the pentraxins (Emsley *et al*., 1994) and certain classes of lectins, despite almost insignificant sequence similarity (10-15%). However, it would be difficult to simply extend the structure-function relationships of the lectins to LG domains. LG domains appear to have a unique multifunctional ligand-binding site distinct from the carbohydrate-binding sites found in lectins**.** Ligand-binding sites in LG-domains differ in location from the carbohydrate-binding sites that have been defined in pentraxins, legume lectins, glucanases and the galectins. Although not strictly superimposable, the carbohydrate-binding sites in the structurally homologous lectins all cluster on the concave face of the β-sandwich (Rudenko *et al*., 2001).

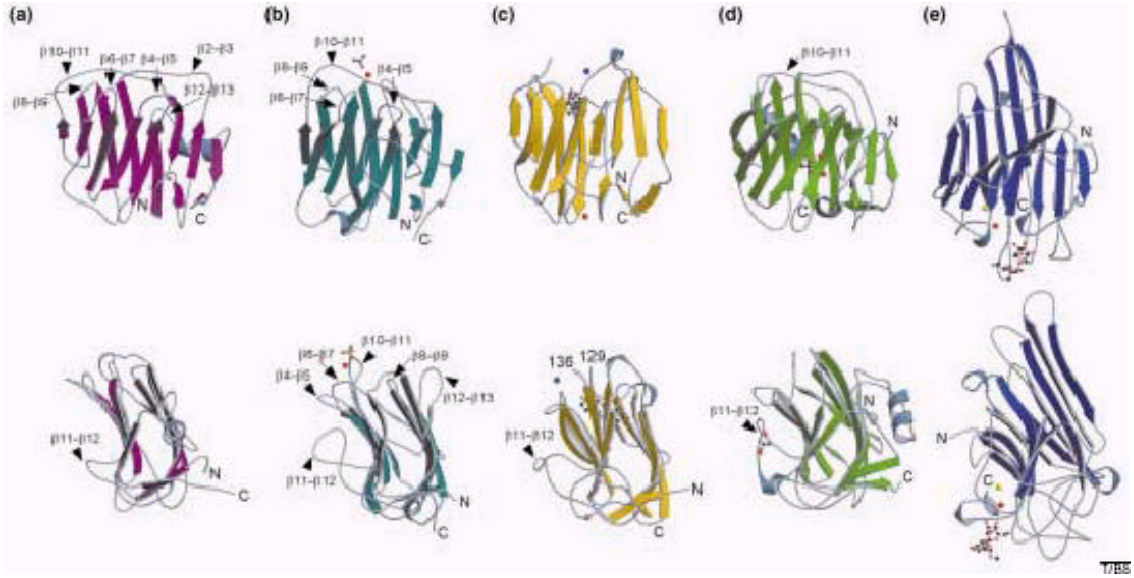

Figure 4.1 Two orthogonal views of the LG domains in (a) the extracellular domain of neurexin 1β (Rudenko *et al*., 1999), (b) domain LG5 of laminin α2 chain (Hohenester *et al*., 1999) and (c) SHBG compared to the jelly roll fold of (d) the pentraxin SAP (Emsley *et al*., 1994) and the legume lectin concanavalin A (Hardman *et al*., 1972). Ca^{2+}ions are shown in red, zinc in blue and magnesium in yellow. Sulfate ions bound to LG5, DHT bound to SHBG and acetate molecule bound at the ligand-binding site of SAP and a disaccharide bound to concanavalin A, are all shown as ball-and-stick models. The figure is taken from the review Rudenko et al (Rudenko *et al*., 2001).

In contrast to the carbohydrate-binding sites in lectins, the crystal structure of SHBG showed that the ligand intercalates in between the two β-sheets at the rim of the β-sandwich. This site, which differs from the ligand-binding sites in lectins, coincides with the ligand interaction site in neurexins and laminin.

Single LG domain of β-neurexins is sufficient for ligand binding, it binds α-latrotoxin and neuroligin (Ichtchenko *et al*., 1995; Sugita *et al*., 1999). An interesting aspect of the binding of neurexins to α-latrotoxin and neuroligin is the tight regulation of these interactions by alternative splicing. This regulatory mechanism is also observed for the interaction of LG domain from agrin with its ligands (Ferns *et al*., 1993). Both sites of alternative splicing in neurexins and agrin when mapped onto the three dimensional structure of the LG domain of neurexin1β are located on the same surface of the LG domain in loops on the edge of jelly roll, opposite to the N-and C-termini. The α2 LG4-LG5 fragment contains major binding

sites for α-dystroglycan, heparin and sulfatides. Binding of α-dystroglycan is strictly calcium dependant (Talts *et al*., 1999). The protein side-chains, which chelate calcium ions, are highly conserved in most LG domains of laminins, neurexins, agrin and perlecan. The wide cleft between the LG4 and LG5 domains contains numerous basic residues, which together with the calcium sites at the extremities of the α2 LG4-LG5 tandem, form an extensive region of positive potential, suitable for binding acidic ligands such as α-dystroglycan and heparin (Tisi *et al*., 2000).

LG domains appear to have a conserved ligand-interaction site at the rim of the β-sandwich opposite the N- and C-termini that, in different proteins, can bind a very wide diversity of ligands including Zn^{2+}, Ca^{2+}, steroids, glycans, proteoglycans and proteins (Rudenko *et al*., 2001).

4.2. Dimerization and steroid binding

Measurements of the steroid-binding capacity and protein concentration of purified SHBG have led to the prevailing opinion that an equimolar relationship exists between SHBG homodimer and steroid ligand (Hammond, 1993), and this has supported the notion that a single steroid-binding site is located within the dimer interface (Petra, 1991). However, our crystal structure data indicated that amino acid residues within dimer interface do not participate in steroid binding, and that each monomer of the SHBG homodimer contains a steroid-binding site.

The structure of SHBG in complex with steroids differs from the structure of the ligand-binding domain (LBD) of the nuclear hormone receptors (Brzozowski *et al*., 1997; Tanenbaum *et al*., 1998). Firstly, in SHBG the steroid intercalates in between two β-sheets, while LBD nuclear hormones receptors are predominantly α-helical proteins. In both classes of proteins the steroid is held in place by two types of contacts: hydrogen bonding at the two ends and hydrophobic contacts along the body of the ligand.

Despite that SHBG and nuclear hormone receptors bind the same substrates, i.e steroid hormones, these two classes of proteins differ in their functions. SHBG is a transport protein for various steroids and, therefore, its steroid-binding pocket adapts to accommodate different ligands. Thus, the displacing of the 3-oxo group of DHT by the hydroxyl group of

estradiol, methoxyestradiol, 17β-DHA and 17α-DHA creates the possibility to make an additional hydrogen bond with the carbonyl oxygen of Val105 and Thr40. The striking structural feature of the steroid transport protein SHBG is the loop region between the residues Pro130 and His136, which covers the steroid-binding pocket. It undergoes conformational changes in different SHBG-steroid complexes. Apparently, this loop regulates the access of the ligands to the steroid-binding pocket of SHBG. The ligand-binding domain of nuclear receptors is very specific and binds only one class of steroid hormone. Thus, the estrogen receptor, which binds estradiol and its analogues, has a glutamate, which accepts the hydrogen bond donated by the 3-hydroxyl group of its ligands. The other steroid receptors, which bind 3-oxo steroids, have a conserved glutamine to accommodate the 3-oxo group of the hormones. The conserved arginine residue in both types of nuclear receptors serves to correctly orient and position the discriminating glutamate or glutamine side chain via water-mediated hydrogen bonds. The side chain of this arginine itself is positioned by a hydrogen bond to the carbonyl of the residue preceding a conserved phenylalanine, which is fixed by its van der Waals contact to the A-ring of the steroid. Thus, the recognition of the 3-oxy function of the hormone is coordinated with a van der Waals contact to its hydrophobic rings. Ligand binding sites of nuclear receptors are large, for example, the absence of the hormone from estrogen receptor would leave a cavity of about 450Å^3. The overall conformation of the steroid binding sites in nuclear hormone receptors not only accounts for the specificity of ligand binding, but allows the steroid hormone to function as the structural core (Tanenbaum *et al*., 1998).

In the structure $\triangle^5$-3-oxosteroid isomerase, which catalyzes a stereospecific isomerization of steroid substrates, the ligand binds in a mixed α and β secondary structure environment. In contrast to the structures of SHBG with 3-oxo steroids, such as DHT and norgestrel, where only a single hydrogen bond is formed involving the hydroxyl group of Ser42, an extensive network of hydrophilic contacts with one arginine residue and two tyrosine residues anchors the steroid *via* the 3-oxo group in the isomerase (Kim *et al*., 1997).

In summary, the observed conformation of the steroid-binding pocket of SHBG is novel and different from other known proteins involving in steroid binding.

4.3. Zinc effect

A partially occupied metal-binding site was observed in the original crystal structure in close proximity to the steroid-binding site together with a disordered loop region between the residues Pro130 and Arg135. To identify this metal binding site the structure of zinc-soaked crystals of SHBG has been solved at the MDC in my absence. This structure was solved using the trigonal crystals I obtained.

The zinc complexed crystal structure of SHBG showed the presence of two zinc-binding sites in addition to the calcium binding site described in the initial crystal structure of SHBG. One of the zinc binding sites lies in close proximity to the steroid-binding pocket. Zinc is coordinated by two nitrogen atoms provided by the side chains of His83 and His136, and two oxygens from the carboxylate group of Asp65.

It was shown that zinc influences the steroid-binding activity of SHBG (Avvakumov *et al*., 2000). The affinity of SHBG for estradiol is reduced in the presence of 0.1-1 mM Zn^{2+}, while its affinity for androgens is unchanged (zinc effect) (**Figure 4.2**). There are large amounts of zinc in the male reproductive tract, especially in locations where SHBG may play a role in regulating the activities of sex-steroids, such as the prostate (Cowan *et al*., 1976; Lahtonen, 1985).

In both the original crystal structure and in the zinc- complexed crystal structure the loop segment between the residues Pro130 and Arg135 is disordered. The crystal structure of the EDTA-soaked crystals and of SHBG in the tetragonal crystal form now allows me to discuss possible reasons for the disordering of the loop segment in the presence of zinc and also to explain the effect of zinc onto the binding affinity of estradiol. Comparing those structures shows that binding of zinc in this region reorients a number of side-chains. Thus, Asp65 is rotated away from the steroid and participates in zinc binding. Furthermore, both His83 and His136 undergo conformational changes and coordinate zinc. The loop segment covering the steroid-binding site becomes disordered (**Figure 4.3**). In the crystal structure of EDTA-soaked crystals there is no hint for a metal ion bound to this region and the loop is well ordered. Obviously, the reorientation of His136 causes the disordering of the loop. Of all dihedral angles ϕ, ψ of residues 127 to 138, which precede and follow the disordered loop

region, only the ψ-values of His136 and Pro137 change by more than 20° between the zinc- and EDTA-soaked structures. The ψ-values of residues His136 and Pro137 change by 40° and 50°, respectively. In the structure of the EDTA-soaked crystals His136 points away from the metal-binding site and the loop region between the residues Pro130 and His136 (**chapter 3.2.2**) becomes ordered. Thus, the orientation of His136 towards the zinc-binding site promotes the disorder in the flap covering the steroid-binding pocket and, is therefore, responsible for the zinc effect.

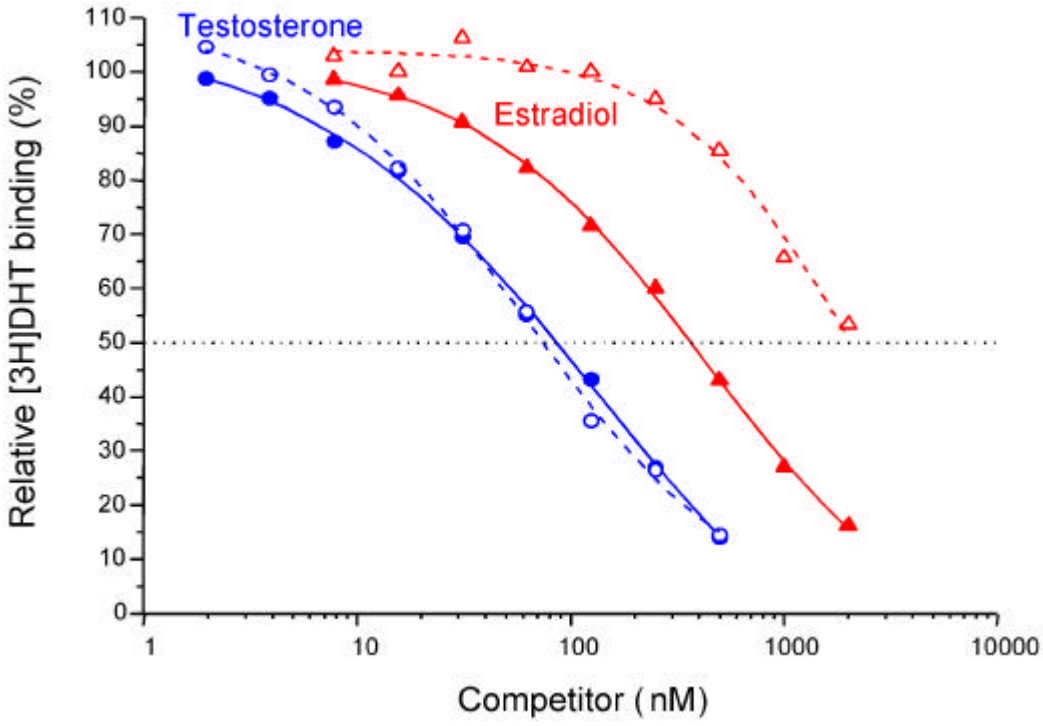

Figure 4.2 Effect of zinc on the competition of androgens and estrogen for the binding of [^{3}H]DHT to purified SHBG. Dose-response curves for the competition of testosterone (circles) and estradiol (triangles) with [^{3}H]DHT for the binding to SHBG at 0°C with (open symbols, dashed lines) or without (solid symbols and lines) preincubation in the presence of 1 mM $ZnCl_2$.

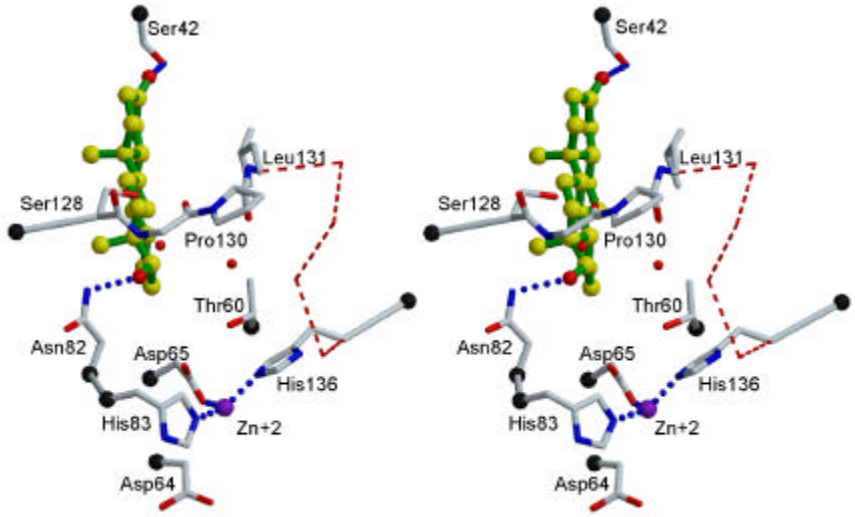

Figure 4.3 Stereo representation of the zinc-binding site in close proximity to the steroid-binding pocket of SHBG. Zinc is coordinated by two nitrogen atoms provided by the side chains of His83 and His136, and two oxygens from the carboxylate group of Asp65. The orientation of His136 towards the zinc-binding site promotes the disorder in the loop covering the steroid-binding pocket (red dashed line) and, apparently, responsible for the zinc effect.