Steroid Ligands Bind Human Sex Hormone-binding Globulin in Specific Orientations and Produce Distinct Changes in Protein Conformation*

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The amino-terminal laminin G-like domain of human sex hormone-binding globulin (SHBG) contains a single high affinity steroid-binding site. Crystal structures of this domain in complex with several different steroid ligands have revealed that estradiol occupies the SHBG steroid-binding site in an opposite orientation when compared with its dihydrotestosterone or C19 androgen metabolites (5α-androstan-3β,17β-diol and 5α-androstan-3β,17α-diol) or the synthetic progestin levonorgestrel. Substitution of specific residues within the SHBG steroid-binding site confirmed that Ser42 plays a key role in determining high affinity interactions by hydrogen bonding to functional groups at C3 of androstenediols and levonorgestrel and the hydroxyl at C17 of estradiol. Among residues participating in the hydrogen bond network with hydroxyl groups at C17 of C19 steroids or C3 of estradiol, Asp65 appears to be the most important. The different binding mode of estradiol is associated with a difference in the position/orientation of residues (Leu131 and Lys134) in the loop segment (Leu131–His136) that covers the steroid-binding site as well as others (Leu171–Lys173 and Trp84) on the surface of human SHBG and may provide a basis for ligand-dependent interactions between SHBG and other macromolecules. These new crystal structures have also enabled us to construct a simple space-filling model that can be used to predict the characteristics of novel SHBG ligands.

Sex hormone-binding globulin (SHBG) is the major plasma transport protein for biologically active sex steroids in a wide range of vertebrate species, and changes in the plasma concentrations of SHBG play a key role in determining the plasma half-lives of its steroid ligands and their access to target tissues (1). The affinity and specificity of SHBG for steroid ligands varies between species, and human SHBG has a high affinity for both testosterone and 17β-estradiol (1, 2) as well as androgen and estrogen metabolites that exert biological effects mediated by nongenomic mechanisms (3). Human SHBG also binds synthetic progestins used in oral contraceptives with close to nanomolar affinities (4) and interacts with several natural and synthetic xenobiotics, albeit with lower affinity (5).

All of these natural and synthetic ligands compete with each other for binding to SHBG, but nothing is known about how they are coordinated within its steroid-binding site.

The realization that the steroid-binding and dimerization domains of human SHBG are located within its amino-terminal laminin G-like (LG) domain (6) prompted us to crystallize this region of the molecule (7) in a complex with 5α-dihydrotestosterone (DHT). This allowed us to visualize the structure of the steroid-binding site (8) and to fully appreciate the results of earlier affinity labeling experiments (9, 10) as well as biochemical studies of recombinant human SHBG variants containing specific amino acid substitutions (11). Subsequent analysis of the amino-terminal LG domain of SHBG has revealed several other features of the molecule that add new dimensions to our understanding of its structure and function. These include a confirmation of the proposed dimer interface and the demonstration that each SHBG monomer contains a functional steroid-binding site (12) rather than a single binding site at the homodimer interface (13). Our crystal structure studies have also led to the discovery that SHBG is a zinc-binding protein and that occupancy of a zinc-binding site alters the specificity of steroid binding (14). This zinc-binding site lies within a loop region that was disordered in our original crystal structure (8): a region of particular interest because it is poorly conserved between species (15) and amino acid substitutions within it influence steroid-binding specificity (11). Resolution of the structure of this extended loop region has recently helped us to understand how zinc influences the conformation and steroid-binding specificity of human SHBG (16).

Because different steroids compete for a single binding site within the SHBG molecule, it has always been assumed that they enter the site in the same orientation. However, there are reasons to believe that particular steroid ligands are coordinated differently within the binding site and may cause specific conformational changes in protein structure. For instance,

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† The abbreviations and trivial names used are: SHBG, sex hormone-binding globulin; LG, laminin G-like; DHT, 5α-dihydrotestosterone; 3β,17β-Adiol, 5α-androstane-3β,17β-diol; 3β,17α-Adiol, 5α-androstane-3β,17α-diol; levonorgestrel, 4-estren-17α-ethylvinyl-18-homo-17β-ol-3-one.
binding of unliganded SHBG to a plasma membrane protein within human prostate cells can be abrogated by occupancy of the steroid-binding site (17), presumably as a result of a change in the conformation of SHBG. In addition, the binding of SHBG to human endometrial plasma membranes has been shown to occur only when the steroid-binding site is occupied by estradiol and not testosterone (18), and this also indicates that ligand-specific alterations in SHBG structure may occur. We therefore set out to address this by producing a series of crystal structures containing a variety of different ligands. As a result, we have discovered that the orientation of C19 steroids and estradiol in the SHBG steroid-binding site is quite different and is associated with ligand-specific changes in SHBG tertiary structure. When considered together with the newly defined structure of the loop that lies above the steroid-binding site (16), these data reveal how SHBG steroid binding specificity is achieved and provide insight into how different steroids gain access to the steroid-binding site.

**EXPERIMENTAL PROCEDURES**

**Crystal Production and Analysis**—The amino-terminal LG domain of human SHBG was expressed, purified, and crystallized as described before (7), except that various steroid ligands were used instead of DHT to saturate the steroid-binding site during all steps. 17β-Estradiol and levonorgestrel were from Sigma, whereas 5α-androstane-3β,17β-diol (3β,17β-Adiol) and 5α-androstane-3β,17α-diol (3β,17α-Adiol) were purchased from Steraloids Inc. Crystals of different complexes of SHBG were obtained within 1 week at 20 °C. This was accomplished by mixing 1 μl of protein solution (13 mg/ml protein, 50 mM Na-HEPES, pH 7.5, 2.5 mM CaCl2) together with 1 μl of reservoir solution (20% isopropyl alcohol, 10% polyethylene glycol 400 in 100 mM Na-HEPES, pH 7.5) and equilibrating against 1 ml of reservoir solution. Steroid ligands were added to the crystallization mixture at the following concentrations. Estradiol, 3β,17β-Adiol, and 3β,17α-Adiol were used at 3 μM, whereas levonorgestrel was used at 10 μM. Crystals of the complexes of SHBG with estradiol and 3β,17α-Adiol were obtained by adding 100 mM CaCl2 to the reservoir, whereas 50 mM CaCl2 was added to the reservoir to obtain SHBG complexes with levonorgestrel. Before flash cooling and to prevent ice formation, the crystals were transferred into a soaking solution containing 30% isopropyl alcohol, 10% polyethylene glycol 400 in 100 mM Na-HEPES, pH 7.5, for 5 min. With the exception of the diffraction data of the complex between SHBG and 3β,17β-Adiol collected on a laboratory rotating anode x-ray source, all data sets were collected at beam lines BW7A (estradiol) and BW7B (3β,17α-Adiol, levonorgestrel) of the EMBL outstation at the DESY synchrotron in Hamburg. Data were reduced either with the program package DENZO/SCALEPACK (19) or with the program XDS for 3β,17β-Adiol (20). The crystal structures were solved by difference Fourier techniques using the protein atomic coordinates of SHBG in complex with DHT from the Protein Data Bank (accession code 1D2S). The models were subjected to several rounds of refinement using standard molecular dynamics and energy minimization protocols in the CNS program (21) followed by manual inspection in the program O (22). Since the conformation of the loop segment (Leu113−His128) covering the steroid-binding site is influenced by the presence of zinc during crystallization (8), we performed two different crystallographic model refinement calculations with and without zinc bound to SHBG in complex with 3β,17α-Adiol or levonorgestrel. The convergence of the refinement improved when a partial occupancy of the zinc-binding site was assumed, and therefore we modeled these two complexes with an occupied zinc-binding site. Water molecules were inserted where appropriate. Target geometry parameters for the ligands were retrieved from the HIC-up server (23) (available on the World Wide Web at x-ray.lmc.uu.se/hicup); estradiol) or generated automatically using coordinates obtained from the Cambridge structural data base (www.ccdc.cam.ac.uk; levonorgestrel, 3β,17α-Adiol) or by merging structural features from molecules deposited with the Cambridge structural data base (3β,17α-Adiol). The refined structures have been deposited with the Protein Data Bank.

**Analysis of Space Restrictions around DHT and Estradiol Bound to SHBG**—The potential to accommodate additional atoms immediately attached to the steroid was evaluated as follows. Gap volumes in the binding pocket were identified with the program SURFNET (24). Steroid models were built with methyl groups attached to stereochemically oriented 17β- and β-orientations of 3β,17β- and 3β,17α-steroids around the steroid. A sphere with a radius of 1.5 Å was drawn at each of these positions, and the ratio was calculated between the number of grid points in the sphere belonging to gap volumes in the structure and the total number of grid points in the sphere. Typically, the gap volumes were sampled on a grid with 0.2 Å step width. This analysis was performed for the structure of SHBG in complex with DHT (8), for EDTA-soaked crystals of the latter (16), and for the crystal structure of SHBG in complex with estradiol. The structure of SHBG in complex with DHT derived from the EDTA-soaked crystals includes the loop segment (Leu113−His128) that covers the steroid-binding site (16) and was used throughout as the reference structure (Protein Data Bank accession code 1DKD).

**Production of Human SHBG Variants**—A cDNA encoding the human SHBG precursor (GenBank accession number NM_001040) was inserted into a pSelect-1 phagemid (Promega) for site-directed mutagenesis (25). The oligonucleotide primer sequences for mutagenesis are listed in Table I. The mutated cDNAs were subcloned into a pBMCV eucaryotic expression vector (Invitrogen) and sequenced to confirm that only the targeted mutations had occurred. The resulting plasmids were transfected into Chinese hamster ovary cells (CHO pro-, wild type) using LipofectAMINE reagent (Invitrogen) according to the protocol suggested by the manufacturer. After selection in the presence of Geneticin (Invitrogen), stably transformed cells were grown to near confluence, washed twice with phosphate-buffered saline to remove fetal bovine serum, and then cultured in either CHO-S-SFM-II (Invitrogen) or HyQ PF-CHO LS (HyClone) medium for 2–4 days. Culture media from Chinese hamster ovary cells expressing wild-type or mutated human SHBG cDNAs were concentrated using Ultrafree 15 de

| Amino acid substitutions | Mutagenic oligonucleotidesa |
|--------------------------|-----------------------------|
| S42A                    | 5′-TCGCTAAGAAGCTGAGGTTTTT    |
| S42L                    | 5′-CTCTAAAGAAGCTGAGGTTTTT    |
| G58A                    | 5′-AGGTTGGTGATCCGATATAAAATAC |
| T60A                    | 5′-TAGGTTGGCATCCTCATA        |
| T65A                    | 5′-TATACAACAGATCCTTGAAG      |
| D65V                    | 5′-TATACAACAGATCCTTGAAG      |
| F67A                    | 5′-GAATCCCAAGAATCCAGTCATCTTAA |
| F67B                    | 5′-GAATCCCAAGAATCCAGTCATCTTAA |
| N92A                    | 5′-GGCCGAGTGCCTGCAGTGG      |
| W94A                    | 5′-AAGCTGGGCCCCGATGATTGCG   |
| M107A                   | 5′-TCCCTCCCAGGGCATGCTC       |
| P130G                   | 5′-TCTCCCTCCCAGGGCATGCTC     |
| L131G                   | 5′-GGATAGGCGTTGGTCCGGCCAGAGCCTGTC |
| L131 + GGSGb            | 5′-ATGGCGTTTGTGGTCCCTGAGATCCTCAGGGCCAGAGC |
| K134A                   | 5′-ATGGGAGGCCGTCCGGTCCAGG   |
| H136Q                   | 5′-CATAGGGGTCGTCGTTGGAG       |
| P137G                   | 5′-GGGAAATTCTTCAAGTCGGCGTTGAGTCG |
| M139F                   | 5′-CGGATCGATCCTTCAAGTCGGCGTTGAGTCG |

a Deviations from DNA sequences complementary to the coding sequences spanning amino acids to be modified are indicated in boldface and underlined.

b Five amino acid residues, as indicated, were inserted carboxyl-terminal to Leu121.

**TABLE I**

Oligonucleotide sequences for site-directed mutagenesis of a cDNA encoding the human SHBG precursor in order to produce SHBG variants with specific amino acid substitutions.
ieces with a 30,000 molecular weight cut-off limit (Amicon) and dialyzed overnight at 4 °C in Slide-A-Lyzers (Sigma) against 20 mM Tris-HCl, pH 7.5, 150 mM NaCl containing 1 mM CaCl2, 1 μM DHT, and 0.05% sodium azide. The electrophoretic properties of the human SHBG variants produced in this way were similar to wild-type human SHBG when examined by Western blotting (12, 26).

Steroid-binding Assays—A standard saturation analysis method (27) employing dextran-coated charcoal as a separation agent and [3H]DHT (42 Ci/mmol; PerkinElmer Life Sciences) as the labeled ligand was used to determine the steroid binding capacity, affinity, and specificity of purified human SHBG (14) or recombinant wild-type human SHBG and human SHBG variants in Chinese hamster ovary cell media after concentration and dialysis, as described above. In all cases, the amount of SHBG in samples for analysis was adjusted so that DHT-binding capacities were within 10% of each other. Equilibrium binding parameters of SHBG and SHBG variants were determined by Scatchard analysis (28), and in all cases the ligand-binding isotherms corresponded to a single class of binding sites. A similar experimental protocol was used to determine the binding affinities of SHBG variants for various steroids relative to DHT (27), and the dose-response curves of the steroid ligands tested were in all cases parallel to that obtained with DHT.

RESULTS AND DISCUSSION

Human SHBG-Steroid Complexes Used for Crystallography Experiments—The steroids used in our crystallization experiments were selected for the following reasons. 3β,17β-Adiol is a major androgen metabolite that binds human SHBG with high affinity (1). However, its 3β-hydroxyl is clearly not as well tolerated as the 3-oxo group of DHT (Fig. 1), and we assumed that comparisons of SHBG structures in complex with 3β,17β-Adiol versus DHT would provide insight into how different functional groups at C3 of the steroid molecule interact with residues in the steroid-binding site. By contrast, 3β,17α-Adiol binds SHBG with very low affinity (Fig. 1), and we used this ligand to determine how the orientation of the hydroxyl at C17 influences steroid binding. Levonorgestrel is a synthetic progestin that binds human SHBG with high affinity (Fig. 1) despite the fact that it has an ethynyl group attached to C17 in an α-orientation and an ethyl group at C13 instead of the methyl group present in most natural steroids (1). Like estradiol, levonorgestrel lacks a C19 methyl group, but its ring A is not aromatic, and we expected that a comparison of SHBG complexes with levonorgestrel and estradiol would provide insight into how structural differences between C19 and C18 steroids influence their accommodation within the SHBG steroid-binding site.

Each SHBG-steroid complex crystallized in space group R32, isomorphously to the human SHBG complex with DHT (8, 16) and with less than 1% variations in the cell axis dimensions (Table II). All of the crystal structures were determined at a resolution of 2 Å or better and refined to convergence. This allowed a detailed description of the interactions between the steroids and SHBG. When crystallized in the presence of 3β,17β-Adiol, 3β,17α-Adiol, levonorgestrel, or estradiol, the main chain atoms of SHBG superimpose with the structure of SHBG in complex with DHT (8, 16) with root mean square deviations of 0.42, 0.42, 0.47, and 0.46 Å, respectively. In these structures, 86–90% of all residues were located in the most favorable regions of Ramachandran plots, and none was located in disallowed regions when compared with a compilation of high resolution crystal structures (29). The average thermal displacement factors were 27.4 Å2 (3β,17β-Adiol), 38.5 Å2 (3β,17α-Adiol), 40.8 Å2 (levonorgestrel), and 29.9 Å2 (estradiol), and these differences probably reflect the quality of the crystals used for data collection rather than a specific property of the different complexes.

C19 Steroids and Levonorgestrel Bind to SHBG in the Same Orientation—Both androstenediols and levonorgestrel bind SHBG in the same orientation as DHT (8, 16). However, unlike the 3-oxo group of DHT, which forms a single hydrogen bond with Ser42 (8, 16), the hydroxyl at C3 of 3β,17β-Adiol (Fig. 2A)
or 3β,17α-adiol (Fig. 2B) can act both as a hydrogen bond acceptor and a donor. As a result, it is capable of hydrogen bonding with the carbonyl oxygen of either Thr40 or Val105, in addition to the hydroxyl of Ser42. Although this 3β-hydroxyl in the androstanediols is only separated from the carbonyl oxygen of Val105 by about 2.9 Å, a hydrogen bond with the carbonyl oxygen of Thr40 is more likely because a peptide flip between Thr40 and Ser41 occurred in both androstanediol complexes, when compared with the DHT complex (8, 16).

The hydrogen bond network at the 17β-hydroxyl involves Asp65 and Asn82 and is identical in SHBG complexes with 3β,17α-adiol (Fig. 2A) and DHT (8, 16). Moreover, the conformation of the loop segment between Leu131 and His136 in the structure of SHBG bound to 3β,17α-adiol is very clearly defined (Fig. 2A). Within this loop segment, which is located right over the steroid-binding pocket, Lys134 contacts the 17β-hydroxyl of 3β,17α-adiol (Fig. 2A), as it does in the crystal structure of SHBG in complex with DHT after metal ions have been removed (16). In the 3β,17α-adiol complex, the α-orientation of the 17-hydroxyl precludes its interaction with the side chain of Asn82, but a hydrogen bond between the 17α-hydroxyl and the side chain of Asp65 is maintained (Fig. 2B). The conformation of the loop segment that covers the steroid-binding pocket was poorly defined in this structure. It appears that the 17α-hydroxyl forces Lys134 to point toward the solvent and abolishes any interaction between the side chain of this residue and the steroid (Fig. 2B).

As in the case of DHT (8, 16), the 3-oxo group of levonorgestrel forms a single hydrogen bond with the side chain of Ser42, and its 17β-hydroxyl interacts with Asp65 and Asn82 (Fig. 2C). The loop segment between residues 131 and 136 is disordered, and it is not clear whether the 17β-hydroxyl of levonorgestrel interacts with the side chain of Lys134. In this case, the space requirements to accommodate the ethynyl group at C17 might prevent the loop segment from properly folding over the steroid-binding site and contribute to its conformational disorder. The fact that the ethyl group at C13 of levonorgestrel fits into a small pocket surrounded by the side chains of residues Phe67, Leu80, Asn82, Val112, Val127, and Ser128 without any rearrangements (Fig. 2C) is remarkable and may contribute to the relatively high affinity of SHBG for this synthetic progestin (Fig. 1).

 Estradiol Binds to SHBG in a Distinct Orientation—When compared with other SHBG-steroid complexes we have characterised, the orientation of the 32089

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**Fig. 2.** Stereo images of the human SHBG steroid-binding site occupied by 3β,17α-adiol (A), 3β,17α-adiol (B), and levonorgestrel (C). The orientation of these ligands within the human SHBG steroid-binding site is similar to that observed previously for DHT (8). Images were prepared with Molscript (34) and Raster3d (35).
Androgens and Estradiol Bind SHBG in Different Orientations

Fig. 3. Estradiol binds to human SHBG in an opposite orientation when compared with 3β,17β-Adiol. A, stereo image of the human SHBG steroid-binding site occupied by estradiol. B, superposition of the steroid-binding site in the 3β,17β-Adiol (in blue) and estradiol (in yellow) complexes, illustrating the differences in steroid orientation and coordination. Images were prepared with Molscript (34) and Raster3d (35).

Some obvious conformational adjustments result from the different orientation of estradiol in the binding site. This is best appreciated by comparing the complexes of SHBG with estradiol and 3β,17β-Adiol (Fig. 3B). In both of these complexes, the loop structure over the steroid-binding pocket (residues 131–136) was clearly visible in the absence of a zinc ion (Figs. 2A and 3A). In the case of estradiol, its α-side packs directly against the side chain of Phe67, and such a close interaction is not possible in the SHBG complex with 3β,17β-Adiol, because the C18 and C19 methyl groups need to be accommodated. As a consequence, estradiol is slightly displaced toward Phe67, when compared with 3β,17β-Adiol (Fig. 3B). On the other side of the estradiol molecule, the C18 methyl group pushes the side chain of Leu171 out from the binding pocket by about 1.1 Å (Fig. 3B). As a result, the main chain atoms of Leu171, Asp172, and Lys173 are displaced by 1.03, 1.30, and 0.97 Å, respectively, and this may be important, because these residues reside on the surface of SHBG (8, 16). By contrast, the side chains of residues Leu131 and Lys134 appear to be drawn into the steroid-binding site when it is occupied by estradiol, and the C-α of these residues are displaced by 0.69 and 1.26 Å, respectively, toward the steroid. In the estradiol complex, the side chain of Lys134 is within hydrogen-bonding distance to Ser128, whereas in the other complexes a water molecule bridged this contact, and this might stabilize the loop segment (residues 131–136) that covers the steroid-binding site. In support of this possibility, we observed that the thermal displacement factors of these residues in the SHBG-estradiol complex are about 10 Å² lower than those of the corresponding residues in the 3β,17β-Adiol complex, whereas the thermal displacement factors of all other residues were very similar (not shown).

In both binding orientations, the steroid is completely buried within the molecule. This raises the interesting question of how ligand binding can influence the recognition of SHBG by other...
proteins, such as a putative plasma membrane receptor for SHBG (18). In addition to conformational changes in the segments Leu131–His136 and Leu171–Lys173 described above, the side chain of Trp84 on the surface of SHBG is rotated outwards in the estradiol complex when compared with the 3β,17βAdiol complex (Fig. 3B), and these changes in SHBG conformation could provide a basis for its ligand-specific interactions with plasma-membrane proteins (18). This 180° rotation around the χ2 dihedral angle of the Trp84 side chain appears to be a direct consequence of the inward movement of the loop segment 131–136. It may also be influenced by spatial restrictions introduced by closer contact between the steroid and the side chain of Lys134 when estradiol is present in the SHBG steroid-binding site. Unfortunately, these observations do not allow us to address the important issue of how unliganded SHBG might interact preferentially with plasma membrane-binding sites (17, 30), and we will not be able to resolve this until an SHBG crystal structure is obtained in the absence of steroid ligands.

**Contributions of Specific Amino Acid Residues to the Affinity and Specificity of SHBG Steroid Interactions**—Based on the crystal structures of various SHBG-steroid complexes, a series of human SHBG variants with specific amino acid substitutions were produced to determine how particular residues contribute to steroid binding. These SHBG variants were produced as recombinant proteins in mammalian cells and function as fully glycosylated, homodimeric proteins in the same way as the protein in human blood (11, 25). In these studies, we compared the binding affinity and specificity of each SHBG variant with those of the wild-type protein (Table III). The steroid-binding specificity of these variants was determined using the most important physiological ligands of SHBG (i.e. testosterone and estradiol), but we also tested the relative binding affinities of SHBG variants for 3β,17βAdiol (not shown) and found that they mirrored binding affinities for testosterone (Table III).

**Roles of Amino Acid Residues That Contribute to Hydrogen Bonding**—To assess how hydrogen bonding contributes to SHBG-steroid interactions, we focused our attention on the residues (Ser42, Asp65, Asn82, and Lys134) that interact with functional groups of steroid ligands (Figs. 2 and 3). As expected, substitution of Ser42 with alanine causes a marked reduction in the binding affinity for both androgens and estradiol (Table III), presumably due to a loss of hydrogen bonding with functional groups at C3 and C17, respectively. However, this substitution had a smaller effect on estradiol binding, which was demonstrated by the increased ability of estradiol to compete with labeled DHT for the binding to the S42A variant as compared with wild-type SHBG (Table III). The reduction in the SHBG steroid-binding affinity was even greater when Ser42 was substituted by leucine, and this probably reflects the additive effect of steric hindrance. In all of the SHBG-steroid complexes we have studied, the side chain of Ser42 donates a hydrogen bond to the steroid. In the complexes with the androstanediols and estradiol, the hydrogen bond acceptor is a hydroxy group, and additional hydrogen bonds can be formed with SHBG (Figs. 2 and 3). In the case of 3β,17βAdiol, the most probable acceptor is the carbonyl oxygen of Thr401, but this does not appear to be as important as the interaction between the carbonyl oxygen of Val105 and the hydroxyl at C17 of estradiol. We therefore conclude that hydrogen bonding between functional groups at C3 (C19 steroids) or C17 (estradiol) and the hydroxyl of Ser42 is of paramount importance for high affinity steroid binding. However, in the case of estradiol, it could be partially compensated for by an alternative interaction in which the steroid functions as a hydrogen bond donor.

All crystal structures we have obtained show that the side chains of Asp65 and Asn82 can participate in the formation of hydrogen bonds with functional groups of the steroid molecule, and this very likely contributes to high affinity interactions. However, the steroid-binding properties of SHBG were only changed when Asp65 was substituted by alanine or valine (Table III). In the case of the D65A variant, the binding of estradiol was almost abolished, whereas the binding affinities for DHT and testosterone remained unchanged. When Asp65 was substituted with more bulky valine, the affinities for C19 steroids were also markedly reduced. Therefore, the hydrogen bond between the side chain of Asp65 and the steroid appears of particular importance for the binding of estradiol. This might also explain why the presence of a zinc ion in this region of SHBG, which causes the side chain of Asp65 to move away from the steroid, only affects the binding of estradiol (14). By contrast, the binding affinities of the N82A variant for both androgen and estradiol were normal. This was surprising, because Asn82 is located sufficiently close to a steroid functional group to allow formation of a hydrogen bond in the SHBG complexes with DHT (8, 16), 3β,17βAdiol (Fig. 2A), levonorgestrel (Fig. 2C), and estradiol (Fig. 3A). It is possible that Asn82 and Asp65 both contribute significantly to the binding of androgens, and when they are substituted individually, the binding affinities for androgens are not reduced, because the remaining interaction compensates. However, as discussed above, the interaction between estradiol and Asp65 is much more important than the interaction of androgens with this residue, and our data imply that in the case of estradiol, loss of this interaction cannot be compensated for by an apparently weak interaction with Asn82.

The T60A variant shows an increase in the affinity for estradiol, whereas the affinities for androgens remain unchanged (Table III). The hydroxy group of Thr401 forms a hydrogen bond with the carboxyl group of Asp65 and thus influences its orientation (8). As is apparent from the binding data, removal of this restraint improves the coordination of estradiol but not of androgens, and this is in line with our conclusions about the relative importance of Asp65 for the binding of these two classes of sex steroids.
Roles of Amino Acid Residues within the Loop Segment (Leu$^{131}$–His$^{136}$) Covering the Steroid-binding Pocket—It has been observed previously that residues within the loop segment (Leu$^{131}$–His$^{136}$) that covers the steroid-binding site are important determinants of the steroid-binding specificity of human SHBG (11), and we have recently shown how they contribute to the high affinity binding of DHT (16). As in the latter report, the binding affinity of the L131G variant for DHT is significantly reduced, and this was even more evident in the case of testosterone (Table III) and 3β,17β-Adiol (not shown). Interestingly, this amino acid substitution had a less marked effect on the binding of estradiol (Table III), and this further emphasizes the differences in how androgens and estrogens interact with residues within this loop segment. As noted previously (11), substitution of His$^{136}$ with Gln led to a specific reduction in the SHBG binding affinity for estradiol (Table III). However, the altered position of His$^{136}$ when a zinc-binding site in this region is occupied causes a marked and specific reduction in estradiol binding (14), and it is therefore possible that a glutamine at this position has an effect on the conformation of the loop segment covering the steroid-binding site. Among other residues in this loop segment, previous reports using affinity labeling techniques have shown that Lys$^{154}$ is located close to the steroid ligand (10), and when this residue was substituted with histidine it again led to a small but specific reduction in the binding affinity for estradiol (11). However, substitution of Lys$^{154}$ with alanine had no effect on steroid binding (Table III), and this indicates that its contribution to the binding affinity of either androgens or estrogens is minimal.

To further explore how the conformation of the loop segment (Leu$^{131}$–His$^{136}$) that covers the steroid-binding site might influence the steroid binding specificity of SHBG, we individually substituted the proline residues (Pro$^{130}$ and Pro$^{137}$) flanking this segment with glycines in order to increase its mobility. When this was done, there was again no effect on androgen binding, but estradiol binding was enhanced (Table III). Since our crystal structure data show that this loop segment is drawn into the steroid-binding pocket in the presence of estradiol (see above), the removal of constraints to its mobility by substitution of the flanking prolines with glycines may facilitate this and specifically increase the relative binding affinity for estradiol. By contrast, the insertion of several residues (Gly–Gly–Ser–Gly) into this loop segment immediately after Lys$^{131}$ had no effect on either the steroid-binding affinity or specificity of SHBG. Albeit unexpected, it is possible that this insertion may have little or no effect on the mobility of the loop as a whole. If so, any movement in the loop segment probably influences the positions of specific residues that are most important for estrogen binding.

Contributions of Hydrophobic Residues Lining the Steroid-binding Pocket—A number of hydrophobic residues line the steroid-binding pocket, and among these, Phe$^{67}$, Met$^{107}$, and Met$^{139}$ contribute the highest amount of contact surface with the steroid. When Met$^{107}$ was substituted with alanine, no effect on the binding of androgens or estradiol was detected (Table III). Although the substitution of Phe$^{67}$ with alanine also had no effect on androgen binding, it increased the SHBG affinity for estradiol (Table III). This is understandable, considering the specific orientation of estradiol in the binding site (Fig. 3B) and its close contact with this residue (see above). As noted earlier (31), substitution of Met$^{139}$ leads to a marked reduction in steroid binding affinity, and the binding of all steroid ligands tested was virtually undetectable when a bulky and particularly hydrophobic residue (Phe) was present at this position (Table III). Thus, it would appear that the size of the residue at this position rather than its hydrophobicity influences the docking of steroids within the binding pocket. Similarly, as predicted previously (8, 16), Gly$^{38}$ is located in such close proximity to the steroid that even its substitution with alanine caused a very marked reduction in the binding affinity for steroids (Table III). In this case, estradiol binding was again somewhat less affected, which is probably explained by its different orientation within the binding site.

Since estradiol binding causes a change in the orientation of
the side chain of Trp$^{84}$ (Fig. 3B), we substituted this residue with alanine, and this greatly improved the binding of estradiol to SHBG but reduced the binding of C19 steroids (Table III). Recent photoaffinity labeling studies with 17α-alkylamine derivatives of β3,17β-alkyl-estradiol have indicated that Trp$^{84}$ is located in the vicinity of the steroid-binding site (32). Although Trp$^{84}$ is not in direct contact with the steroid, our data suggest that Trp$^{84}$ contributes to high affinity binding of androgens. The increased binding affinity of the W84A variant for estradiol can be explained when assuming an energetically less favorable conformation of Trp$^{84}$ in the estradiol complex.

Predictions of SHBG-Ligand Interactions—Simple space availability considerations allow us to predict how other steroids may be accommodated in the binding site. This was done to test the feasibility of identifying novel ligands for SHBG that could be used to further explore its role in regulating steroid hormone action. Accordingly, in our model calculations, we attached methyl groups in stereochemically correct α- and β-orientations to the steroid carbon atoms. Although binding constants for only a few of these methyl-substituted steroids have been determined experimentally (1), these values compare favorably with our prediction of space availability (Fig. 4). For instance, space is available at the position 1α when the loop segment Leu$^{131}$–His$^{136}$ is disordered (Fig. 4A), but this is not the case for position 2α. In accordance with this, 1α-methyl-DHT binds to SHBG with high affinity, whereas 2α-methyl-DHT binds poorly (1). Limited space is also available at the position 7α, and 7α-methyl-DHT and 7α-methyl-testosterone bind to SHBG with rather high affinities (1). In addition, we predict that 4α-methyl-DHT will bind to SHBG with considerable affinity. Our model calculations further show that space is available in the SHBG-DHT complex for an additional methyl group attached to atom C18, and its preferred orientation would correspond to that observed in the SHBG-levonorgestrel complex. Although limited space is available for a methyl group in the α-orientation at C17 when the loop segment Leu$^{131}$–His$^{136}$ is ordered, considerably more space is accessible when this loop is disordered. Since SHBG binds 17α-ethyl DHT with very high affinity (1), this also suggests that the loop structure covering the steroid-binding site is only loosely ordered in unliganded SHBG.

Since estradiol is oriented quite differently within the SHBG-steroid-binding site, we performed a separate analysis of space-filling restrictions using the SHBG structure in complex with estradiol. In this case, a limited number of positions may accommodate an additional methyl group (Fig. 4B). For example, functional groups may be added at C2 and C4 of estradiol, and it is known that 2-methoxyestradiol is a potent ligand for SHBG (1). These studies also allow us to predict that estradiol derivatives with additions at C15 represent novel ligands of human SHBG.

Although the considerations presented here do not allow for a detailed prediction of the binding behavior of potential SHBG ligands, they highlight that simple considerations of space availability allow the behavior of several closely related steroids to be understood. Finally, our observation that androgens and estradiol bind to SHBG in different orientations cautions against the use of homologous modeling (33) and highlights the necessity for experimentally determined crystal structures.

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