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# Structure/function analyses of human sex hormone-binding globulin: effects of zinc on steroid-binding specificity☆

Geoffrey L. Hammond[a,*], George V. Avvakumov[a], Yves A. Muller[b]

[a] *Departments of Obstetrics & Gynecology and Pharmacology & Toxicology, Canadian Institutes of Health Research Group in Fetal and Neonatal Health and Development, The University of Western Ontario, London, Ont., Canada N6A 4L6*

[b] *Department of Biochemistry, School of Biological Sciences, University of Sussex, Brighton BN1 9QG, UK*

**Abstract**

In humans, sex hormone-binding globulin (SHBG) binds and transports the biologically most important androgens and estrogens in the blood, and regulates the access of these steroids to their targets tissues. In addition to binding sex steroids, SHBG has specific binding sites for divalent cations including calcium and zinc. Zinc binding to a site at the entrance of the steroid-binding pocket in human SHBG has been shown to reduce its affinity for estrogens, while having no impact on the binding of C19 steroids. Crystallographic studies indicate that C18 and C19 steroids are bound in opposite orientations within the SHBG steroid-binding site, and we have obtained new information that supports a molecular model explaining the mechanism by which zinc alters the affinity of human SHBG for estrogens, by studying directly the estradiol-binding properties SHBG variants created by site-directed mutagenesis. In this model, the coordination of a zinc ion by the side chains of residues Asp65 and His136 eliminates a critical hydrogen bond between Asp65 and the hydroxyl at C3 of estrogens, such as estradiol and 2-methoxyestradiol, and causes disorder in a polypeptide loop segment that covers the steroid-binding site. The combination of these structural changes explains the specific decrease in the affinity of human SHBG for C18 steroids in the presence of a zinc ion.


*Keywords:* Crystallography; Steroid-binding site; Site-directed mutagenesis; Amino acid substitution; Divalent metal ions

## 1. Introduction

Biologically active androgens and estrogens are transported in the blood by a plasma glycoprotein, known as sex hormone-binding globulin (SHBG), which is produced in the liver. Because C19 and C18 sex steroids bind human SHBG with high (approximately nM) affinity, its plasma concentrations determine the relative amounts of these hormones that are bound to SHBG versus those that are albumin-bound or in the non-protein-bound (free) fraction in the blood [1]. This is important because SHBG-bound steroids are considered to be unable to leave the blood vessels in target tissues, and only free steroids are thought to enter tissues and gain access to target cells [1,2]. On the other hand, specific pathways through which SHBG-bound steroids might exert their hormonal activities have also been suggested [3].

In addition to natural steroids, human SHBG binds several pharmaceutically important synthetic steroids including many progestins used extensively in contraceptive formulations and, more recently, as components of hormonal replacement therapies. It has therefore been important to gain a deeper understanding of the modes of ligand binding within the human SHBG steroid-binding site. To accomplish this, we have recently undertaken a series of crystallographic and structure/function studies involving the production and analysis of various recombinant forms of human SHBG and their molecular variants [4–9]. The following summarises our most important findings in the context of prevailing opinions of how this protein regulates the access of its steroid ligands to their target cells. We also present new data that have enabled us to refine a molecular model that explains more definitively how zinc binding to human SHBG affects its relative affinity for estrogens as compared to androgens.

## 2. Crystal structure of human SHBG

Shortly after its primary structure had been solved by protein sequencing [10] and molecular cloning [11] efforts, it

*Abbreviations:* DHT, 5α-dihydrotestosterone; LG, laminin G-like; SHBG, sex hormone-binding globulin

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* Corresponding author. Present address: B.C. Research Institute for Children's and Women's Health, 950 West 28th Avenue, Vancouver, BC, Canada V5Z 4H4.

*E-mail address:* ghammond@cw.bc.ca (G.L. Hammond).

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was recognised that human SHBG comprises of two laminin G-like (LG) domains [12]. Furthermore, the realization that SHBG did not require glycosylation to form homodimers or a functional steroid-binding site [13] led to the successful production of a series of SHBG deletion mutants in *E. coli*, some of which retained appropriate steroid-binding properties [14]. These studies were also important because they demonstrated that the steroid-binding and dimerization sites of SHBG reside within its amino-terminal LG domain [14], and provided sufficient amounts of recombinant proteins for crystallisation [15] of the amino-terminal LG domain of human SHBG in complex with the androgen 5α-dihydrotestosterone (DHT). These crystals not only provided insight into how this ligand is co-ordinated within the steroid-binding site but also revealed that each monomer of the SHBG homodimer comprises a steroid-binding site [4]. The latter came as a surprise since it had previously been assumed that the SHBG homodimer contains only a single steroid-binding site located between the two subunits [16,17]. Subsequent studies involving the production of dimerization-deficient SHBG variants verified the location of dimer interface between the subunits and confirmed the presence of two functional steroid-binding sites per homodimer [6].

Although the structure of a complete human SHBG monomer has not yet been resolved, the predicted quaternary structure of the homodimer [4] is consistent with the general shape and dimensions of human SHBG, as determined by size-exclusion chromatography and its appearance on electron micrographs [18]. Furthermore, the recent crystal structure determination of the C-terminal pair of LG-like domains of the growth arrest-specific protein Gas6 [19], which are often referred to as the SHBG-like domain of this and other related proteins [20], should help refine predictions of how the two LG domains of SHBG associate with each other.

## 3. Orientation and coordination of ligands within the human SHBG steroid-binding site

Numerous early studies [21] provided information about the affinity and specificity of human SHBG for biologically active androgens and estrogens, as well as a variety of synthetic steroids and low-molecular-weight compounds, including drugs [22]. These data helped identify functional groups attached to specific carbon atoms in the steroid backbone that are essential for high affinity binding. Thus, since androstenedione and estrone interact with human SHBG very poorly, it was concluded early on that a hydroxyl at C17 of both androgens and estradiol was a key determinant of high affinity binding. Although these data supported the concept that different steroid classes entered the steroid-binding site of SHBG in the same orientation, the binding properties of affinity resins comprising steroid ligands linked to a solid matrix through different functional groups cast some doubt upon this. For instance, affinity resins produced using derivatives of DHT at both C3 and C17 positions have been used successfully to purify human SHBG [21]. Our recent crystal structure data [8,9] have helped resolve this issue because they show that androgens and estrogens are located in the binding site in opposite orientations (Fig. 1). Although it is still possible that C19 and C18 steroids can

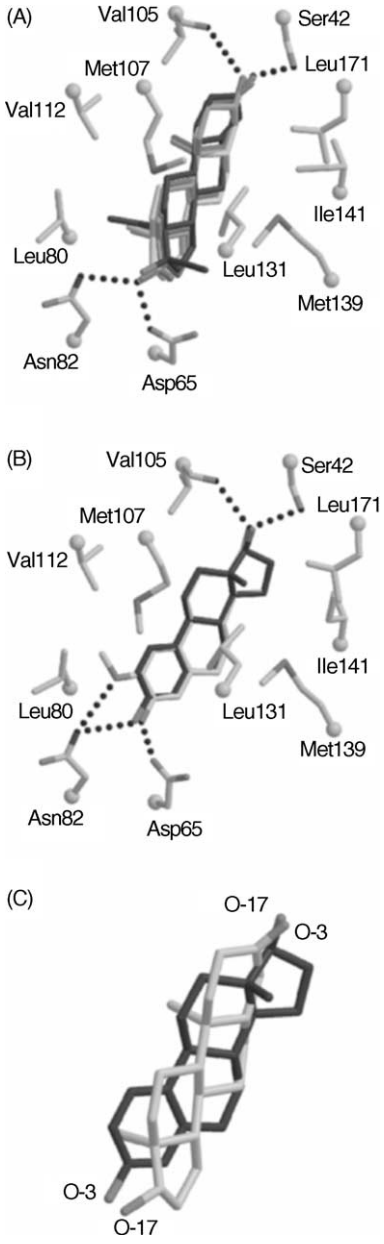


Fig. 1. (A) Steroid-binding site of SHBG occupied with the androgens DHT, 5α-androstane-3β,17β-diol and 5α-androstane-3β,17α-diol (in different shades of gray), and the synthetic contraceptive levonorgestrel (in black). Important hydrophilic interactions between the steroids and SHBG are highlighted with dash lines. (B) Superposition of the estrogens estradiol (in black) and 2-methoxyestrastradiol (in light gray) when bound to SHBG. (C) The superposition of the steroids DHT and estradiol reveals that androgens and estrogens bind to SHBG in opposite direction. Thus, ring A of the C19 steroids and ring D of estradiol reside most deeply within the binding site.

enter the binding site in both orientations, our crystallographic data have revealed that functional groups at C3 of C19 steroids and the 17β-hydroxyl of estradiol hydrogen bond with Ser42 [8]. Furthermore, site directed mutagenesis experiments have shown that this is the most important interaction that contributes to the high affinity of human SHBG for these ligands [8]. The way that steroids gain access to the site remains to be determined but we have obtained evidence that a flexible loop region between Leu131 and His136 may play a role in regulating steroid entry into or efflux from the binding site [7].

## 4. Influence of $Zn^{2+}$ on the binding of estrogens to human SHBG

We have recently discovered that zinc ions reduce the ability of estradiol [5] and 2-methoxyestradiol [9] to compete with [$^3$H]DHT for binding to human SHBG. Based on the location of the two coordination sites for zinc ions in the amino-terminal LG domain of human SHBG [5], we used site-directed mutagenesis to produce an SHBG variant (H136Q) in which one of these zinc-binding sites was disrupted [5]. This demonstrated that the coordination of zinc at this site is responsible for marked changes in the affinity of SHBG for estrogens.

In the previous experiments, the steroid-binding specificity of SHBG variants was assessed in the presence and absence of zinc using [$^3$H]DHT as the labelled ligand in a conventional competitive steroid-binding assay [5]. The results of these studies allowed us to propose a mechanism for the inhibition of estrogen binding to SHBG by zinc ions [5]. However, this did not take into account the inverted orientation of estrogens in the SHBG steroid-binding site as compared to androgens because this was not known at that time. In view of this new information, we now describe experiments designed to study directly the effect of zinc ions on [$^3$H]estradiol binding to wild-type SHBG and SHBG variants in which amino acid substitutions have been introduced at positions known to be important for steroid binding.

In these experiments, the concentrations of the SHBG variants were adjusted so that they demonstrated equal binding capacities as measured by [$^3$H]DHT saturation assay, after the necessary corrections were made to account for their different affinities for DHT [23]. As a result, the observed differences in the amounts of [$^3$H]estradiol bound specifically to different SHBG variants (under standard conditions of equilibration with [$^3$H]estradiol, and subsequent treatment with dextran-coated charcoal to remove unbound steroid), reflected differences in the rates of [$^3$H]estradiol dissociation from the complexes with different SHBG variants, as a measure of their relative affinities for estradiol.

As shown in Fig. 2, none of the amino acid substitutions introduced into the SHBG molecule increased the effect of zinc on estradiol binding to SHBG. Moreover, the ability of zinc to modulate estradiol-binding affinity of a number of the SHBG variants studied was significantly reduced (Fig. 2). Thus, the presence of 0.5 mM zinc had no effect on the apparent affinity of the H136Q, D65A and G58A SHBG variants for estradiol, while the zinc effect was significantly reduced in the case of the P137G, P130G and N82A SHBG variants. Our crystallographic investigations, which lead to the atomic structures of SHBG in complex with DHT in the presence and absence of zinc, provided insight into the structural rearrangements that occur within the steroid-binding site when zinc binds to SHBG. When considered in the context of the crystal structure of SHBG–estradiol complex [8], these data provide for a simple structural model that helps explain the effect of zinc on the binding of estradiol to SHBG (Fig. 3).

The binding of zinc results in a reorientation of the side chain of Asn65 away from the steroid-binding site. When zinc is absent, Asn65 participates in a hydrogen bond with the oxygen atom at C3 of estradiol (Fig. 3A) and, as shown by site directed mutagenesis (Fig. 2), this interaction is a hallmark of the high affinity binding of estradiol to SHBG. When zinc binds to SHBG, Asn65 participates in the coordination of this ion and is no longer capable of the interaction with the steroid (Fig. 3B). In addition to Asn65, binding of zinc reorients the side chain of His136. In the absence of zinc, the side chain of His136 points away from the surface of SHBG (Fig. 3A). Upon binding of a zinc ion at this location of the SHBG molecule, His136 turns inwards and together with Asn65 participates in the coordination of zinc (Fig. 3B). This inward orientation of His136 has a dramatic effect on the structure of the peptide segment formed by residues 130–135. In the absence of zinc, this loop segment forms what can be described as a lid that covers the steroid-binding site. In the presence of zinc, this loop segment loses its well-defined conformation and becomes disordered. We have previously suggested that His136 acts as a lever in this regard, and that disordering of the loop segment 130–135 is an immediate consequence of the reorientation of the side chain of this residue [7]. We believe that loss of interaction between Asp65 and the steroid together with a disordering of the polypeptide loop segment covering the steroid-binding site, which is caused by the reorientation of the side chain of His136, are both responsible for the reduction of the binding affinity of SHBG for estradiol in the presence of zinc.

In accordance with the mechanism outlined above, the relatively low estradiol-binding affinity of the D65A SHBG variant is likely caused by the loss of the interaction between the carboxylic group of Asp65 and atom O3 of estradiol. Adding zinc to this SHBG variant does not lead to any further reduction in its affinity for estradiol (Fig. 2) because zinc cannot bind to the metal-binding site in this location if the side chain of Asp65 is absent. In the case of the H136Q SHBG variant, its estradiol-binding affinity is identical to that of wild-type SHBG. This SHBG variant is not able to bind zinc, and its affinity for estradiol remains unchanged whether zinc is present or not (Fig. 2).

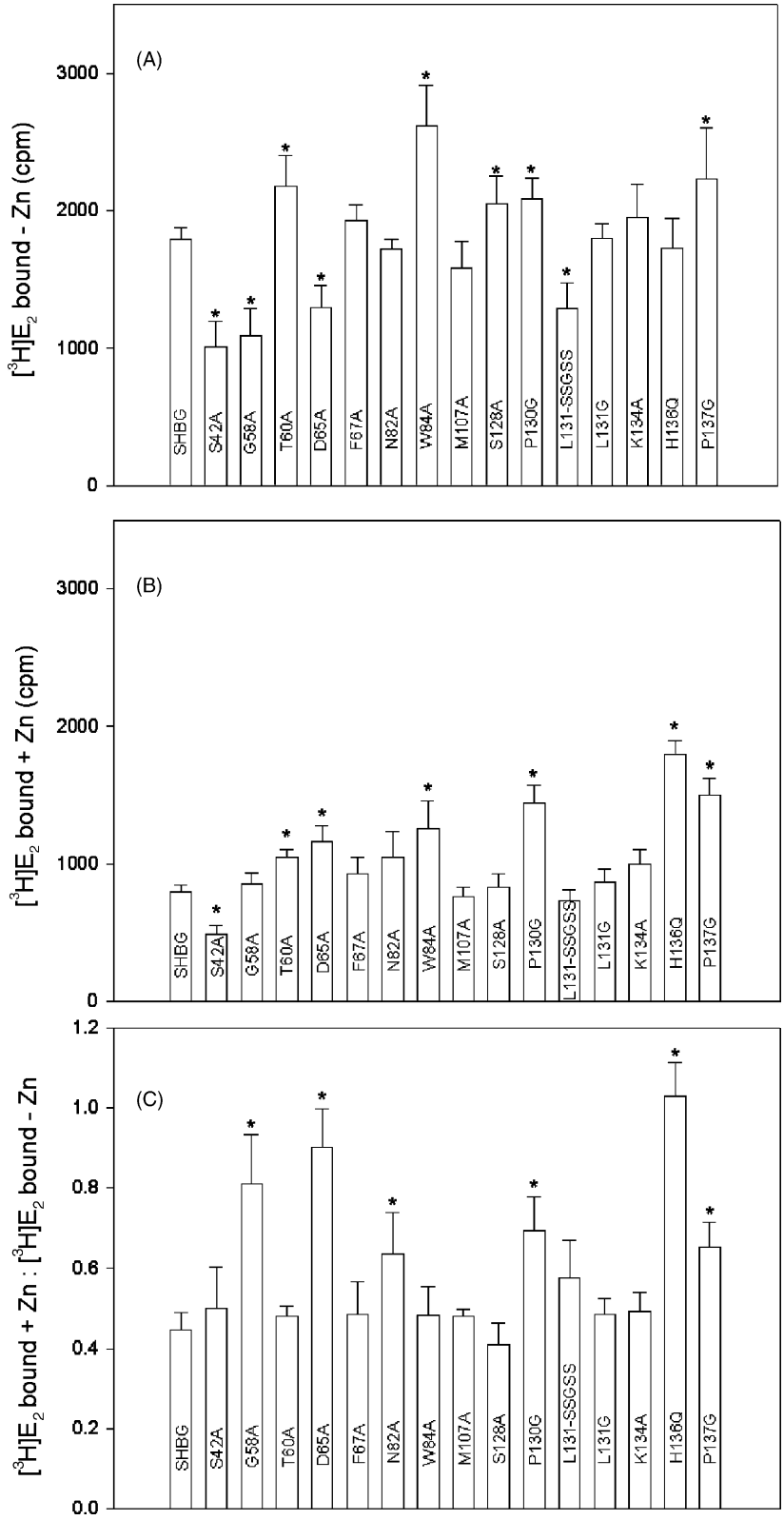


Fig. 2. Influence of $Zn^{2+}$ on the binding of [$^3$H]estradiol ([$^3$H]$E_2$) to human SHBG. A conventional binding assay employing dextran-coated charcoal as a separation agent [23] was slightly modified as described previously [5]. To avoid precipitation and maintain a desired concentration of zinc ions, the assay was conducted in a Tris-buffer [5]. In addition, the time of incubation with the separation agent was reduced to 5 min to minimize dissociation of the SHBG-bound estradiol. Panels A and B show specific binding of [$^3$H]$E_2$ in the absence of zinc ions and in the presence of 0.5 mM $ZnCl_2$, respectively. Panel C shows the amounts of [$^3$H]$E_2$ specifically bound to SHBG variants in the presence of zinc ions as ratio of those obtained in the absence of zinc. Asterisks indicate significant ($P < 0.05$) difference from the corresponding data for wild-type SHBG.

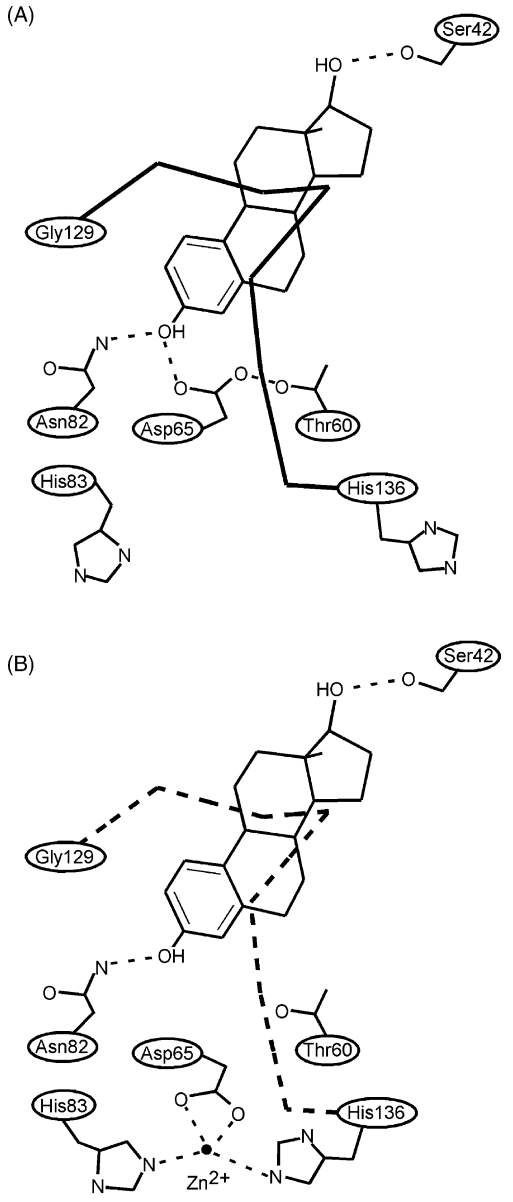


Fig. 3. Structural model illustrating the mechanism for the selective inhibition of estrogen binding to SHBG by zinc ions. Panel A: steroid-binding site in the absence of zinc. The hydrogen bonds between the oxygen atom of the hydroxyl at C3 of estradiol and the side chains of Asp65 and Asn82 are shown (dashed lines). The loop segment 130–135 flanked by residues Gly129 and His136 is indicated by a continuous line. Panel B: the binding of zinc in close proximity to the steroid-binding site leads to a reorientation of the side chains of Asp65 and His136. As a consequence, the interaction between Asp65 and the steroid is abolished and at the same time His136 acts as a lever and the loop segment 130–135 becomes disordered (bold dashed line).

Significantly reduced zinc sensitivity of estradiol binding is also observed for mutants G58A, N82A, P130G and P137G. Addition of a methyl group to the C-α atom of residue 58 most likely produces a steric clash with the steroid. Steric exclusion might therefore prevent the steroid from entering the binding site as deeply as in the case of wild-type SHBG. As a consequence of this, the interaction of the steroid with Asp65, or the closure of the loop segment that acts as a lid, might be disturbed so that binding of zinc to Asp65 and His136 has no further effect on the relatively low steroid-binding affinity of the G58A variant. Although the effect of zinc on mutant N82A is less clear, mutations P130G and P137G which frame the lid segment on both sides most likely have a direct effect on the conformational adaptability of the latter. The increase in the flexibility might allow for the proper closure of the lid segment even when His136 rotates inwards in order to participate in zinc binding.

## 5. Conclusions

The physiological significance of the effect of zinc on the binding of estrogens to human SHBG is not clear. It does not appear to be a highly conserved feature of SHBGs from different mammalian species [5], but could influence the availability of sex steroids to their target cells in human tissues that are known to be rich in zinc, such as the prostate and other male reproductive organs. In addition, exogenous zinc might also influence the plasma distribution of estrogens including 2-methoxyestradiol in the blood circulation, and this phenomenon might be exploited pharmacologically.

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