Direct evidence for the localization of the steroid-binding site of the plasma sex steroid-binding protein (SBP or SHBG) at the interface between the subunits

LI-MING SUI, WILLIAM HUGHES, AGNES J. HOPPE, AND PHILIP H. PÉTRA

Department of Biochemistry, University of Washington, Seattle, Washington 98195 USA

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Abstract

Complete dissociation of dimeric plasma sex steroid-binding protein (SBP or SHBG) was obtained in 6 M urea at 10 °C. Removal of urea resulted in the refolding of monomers, followed by reformation of dimeric SBP, which migrates with the same mobility as the native protein. Dimerization does not require Ca^{++} or steroid. Renatured monomers yield dimers with dissociation constants for 5 α -dihydrotestosterone (DHT) and 17 β -estradiol (E_2) indistinguishable from those of native human SBP. This phenomenon was also demonstrated by mixing human and rabbit SBPs that, upon renaturation, form a hybrid dimer composed of one human subunit and one rabbit subunit. The hybrid binds both DHT and E_2 in contrast to rSBP, which only binds the androgen. Therefore, we conclude that (1) docking of the two subunits creates an asymmetric steroid-binding site located at the interface between the subunits, and (2) only one face of the dimer defines the specificity for binding E_2 by encompassing portion of a structural motif that recognizes the flat ring A of E_2 . The remaining portion, which recognizes the saturated ring A of DHT, is shared by both faces of the dimer. Because native monomers do not exist alone, the often-asked question of whether the SBP monomer binds steroid can be considered meaningless; steroid-binding activity is expressed only in the dimeric state. Finally, formation of the hybrid indicates that SBP dimerization represents a conserved event during the molecular evolution of SBP, suggesting that the structural elements responsible for dimerization will be homologous in SBPs from other species.

Keywords: denaturation; dissociation; 17 β -estradiol; plasma; sex hormone-binding globulin, hybrid, monomeric; sex steroid-binding protein; testosterone

The sex steroid-binding protein (SBP; or sex hormone-binding globulin, SHBG) is a 93.4 kDa protein that specifically binds testosterone (T) and 17 β -estradiol (E_2) in the plasma of many species, including humans (Westphal, 1986; Petra, 1991; Joseph, 1994). The protein controls the plasma metabolic clearance rates of T and E_2 (Petra et al., 1985; Plymate et al., 1990) and is thought to interact with a specific membrane receptor in responding cells, leading to the formation of a steroid ternary complex (Hryb et al., 1985; Fortunati et al., 1991). Although the function of this complex is not yet clear, it is believed to assist cell uptake of the hormone either by endocytosis or by accelerating its diffusion (Bordin & Petra, 1980; Strel'chyonok et al., 1984; Hryb et al., 1985; Gerard, 1995). More recently, however, formation of the ternary E_2 /SBP/SBP-receptor complex has been found to increase the intracellular cAMP concentration in cancer cells that respond to sex steroid hormones, leading to the hypothesis that the complex may function in signal transduction (Rosner et al., 1991; Fissore et al., 1994).

This interesting but controversial proposal suggests that sex steroid hormones could regulate gene expression in two ways: first, through the classic steroid receptor mechanism, and second, by regulating steroid receptors through signal transduction. Recent findings on the phosphorylation of the estrogen receptor (Kato et al., 1995) indicate that cross-talk may indeed exist between the two pathways. However, proof for the involvement of SBP in this process will require cloning and characterization of the SBP receptor.

Biochemical characterization of human SBP performed in the 1970s revealed that it is a glycoprotein dimer that binds one mole of 5 α -dihydrotestosterone (DHT) or T with K_d s of approximately 0.4 nM and 1 nM, respectively, or E_2 with a K_d of approximately 10 nM (Petra, 1979). Rabbit SBP, on the other hand, binds only the androgens with similar K_d s as human SBP (Mickelson & Petra, 1978; Suzuki et al., 1981; Kotite & Musto, 1982). Determination of the amino acid sequences of human and rabbit SBPs by automated Edman degradation, mass spectrometry (Walsh et al., 1986; Griffin et al., 1989), and other structural studies (Turner et al., 1984; Petra et al., 1986a) led us to suggest that the dimeric protein contains identical subunits and that one mole of steroid ligand is bound per dimer. This molecular arrangement suggested that the

Reprint requests to: Philip H. Pétra, Department of Biochemistry, University of Washington, SJ-70, Seattle, Washington 98195; e-mail: hue@u.washington.edu.

steroid-binding site is located at the interface between the subunits. Evidence to support this latter structural feature, however, has been lacking. In order to provide it, we have explored the possibility that SBP could be reversibly denatured in urea and refolded to an active conformation after removal of the denaturant. If this manipulation were to yield monomers, denaturation followed by renaturation of an equimolar mixture of human and rabbit SBPs should lead to the formation of a hybrid dimer composed of one subunit of each. The steroid-binding properties of this hybrid when compared with that of native hSBP and rSBP should shed light on whether the site is located at the interface between the subunits. It should be possible to purify the hybrid by native gel electrophoresis because homodimeric human and rabbit SBPs have markedly different isoelectric points. In this paper, we report the formation, purification, and partial characterization of the hSBP/rSBP hybrid, and we describe its steroid binding specificity leading to the identification of a new structural feature of the steroid-binding site.

Results

Denaturation of hSBP in urea

Human SBP was denatured in increasing concentrations of urea (0–6 M) at 10 °C for 2 hours, and each sample was electrophoresed in polyacrylamide tube gels polymerized in the corresponding concentration of urea. The main results, shown in Figure 1, indicate a progressive loss of native SBP with increasing urea concentration (lanes 1–3), and the concomitant formation of a new band with reduced electrophoretic mobility (lanes 2–5). Complete conversion of native SBP into the new band was achieved in 6 M urea (lane 5), and removal of the denaturant resulted in restoration of the native SBP band (lane 6). Although the monomer has half the molecular mass of native dimeric SBP, its slower electrophoretic migration in native gels is indicative of an increase in the Stokes radius, resulting from unfolding of the subunit to a random coil. The electrophoretic data therefore indicate that urea treatment

Fig. 1. Formation of the hSBP monomer in urea and renaturation to the native hSBP dimer following removal of denaturant. The six lanes represent Coomassie blue stained cylindrical gels electrophoresed in varying concentrations of urea. Human SBP (7–10 μ g) was denatured at various urea concentrations for 2 hours at 10 °C as described in Materials and methods. Lane 1, native hSBP; lane 2, 2 M urea; lane 3, 3 M urea; lane 4, 4 M urea; lane 5, 6 M urea; lane 6, hSBP denatured in 6 M urea and renatured by dilution and dialysis.

Fig. 2. Formation of the hSBP/rSBP hybrid. Samples of hSBP and rSBP (7–10 μ g) were treated or not treated with urea individually or mixed, renatured, and applied to a native polyacrylamide slab gel as described in Materials and methods. The gel was stained with Coomassie blue. Lane 1, native hSBP; lane 2, native rSBP; lane 3, mixed native hSBP and rSBP but untreated; lane 4, hSBP treated with 6 M urea and renatured; lane 5, rSBP treated with 6 M urea and renatured; lane 6, equimolar mixture of hSBP and rSBP treated with 6 M urea and renatured.

under the stated conditions induces a reversible structural change in the SBP molecule, likely resulting in the dissociation of its subunits.

Formation of the hSBP/rSBP hybrid

In order to prove that the new slower-migrating band in Figure 1 represents the SBP subunit, we show in Figure 2 that it represents the intermediate step in renaturation to the dimeric state by mixing human and rabbit SBPs in equimolar concentration, denaturing in 6 M urea, and renaturing by dilution and dialysis. Lanes 1 and 2 are controls indicating that the rSBP dimer has a higher electrophoretic mobility in native gels than the hSBP dimer, as previously found (Petra et al., 1983). Lane 3 demonstrates the migration of a mixture of hSBP and rSBP incubated in the absence of urea. Lanes 4 and 5 represent hSBP and rSBP individually denatured in 6 M urea and renatured by dialysis; no difference in mobility can be detected between the native and renatured proteins. Lane 6, however, shows the presence of a new band that migrates between hSBP and rSBP. This band is absent in lane 3 when the two proteins are mixed in the absence of urea prior to electrophoresis; the new band therefore represents the hSBP/rSBP hybrid, and its formation occurs only when the mixture of hSBP and rSBP is treated with urea.

Direct evidence for the presence of hSBP and rSBP subunits in the hybrid

To demonstrate that the hybrid is composed of one subunit of hSBP and one subunit of rSBP, biotin-streptavidin and immunochemical methodologies were used. Figure 3A shows a native electroblot probed with streptavidin and biotinylated alkaline phosphatase. Lane 1 is a biotinylated-rSBP positive control synthesized as described in Materials and methods. Lane 2 is a hSBP negative control that would not be expected to stain with streptavidin and biotinylated alkaline phosphatase. The sample in lane 3 is an equi-

Fig. 3. Detection of rSBP and hSBP monomers in the hSBP/rSBP hybrid. **A:** Native electroblot probed with streptavidin and biotinylated alkaline phosphatase. Lane 1, biotinylated rSBP (500 ng); lane 2, native hSBP (500 ng); lane 3, unpurified equimolar mixture of hSBP and biotinylated SBP treated with 6 M urea and renatured by dilution and dialysis. **B:** Electroblot probed with anti-hSBP and rabbit anti-goat IgG coupled with alkaline phosphatase. Lane 1, pure hSBP/rSBP hybrid; lane 2, native hSBP (500 ng).

molar mixture of hSBP and biotinylated-rSBP treated with 6 M urea and renatured by dilution and dialysis. The top band represents the hSBP/biotinylated-rSBP hybrid, which has essentially the same mobility as the hSBP/rSBP hybrid (lane 6 of Fig. 2). Positive staining of that band with biotinylated alkaline phosphatase indicates the presence of the biotinylated rSBP monomer in the hybrid. Because the sample applied to lane 3 of Figure 3A was not purified, the lower band represents biotinylated rSBP dimer that reforms during renaturation. Figure 3B represents an electroblot of the purified hybrid and renatured hSBP probed with goat anti-hSBP and rabbit anti-goat IgG coupled with alkaline phosphatase. The band in lane 1 that migrates faster than hSBP (lane 2) is the hSBP/rSBP hybrid; it has the same mobility as the hSBP/biotinylated rSBP hybrid (lane 3, Fig. 3A) and the middle band of lane 6 in Figure 2. Staining of the band in lane 1 of Figure 3B thus reveals the presence of the hSBP monomer in the hybrid.

Effects of calcium and DHT on SBP dimerization

Pure rSBP was denatured in 6 M urea, treated with dextran-charcoal, and renatured by diluting with buffers containing EDTA or CaCl_2 in the presence or absence of DHT. The electrophoretic pattern of Figure 4 indicates that dimerization of SBP occurs whether or not Ca^{++} or DHT is present during renaturation. We conclude that Ca^{++} and DHT do not play a role in dimerization.

Characterization and steroid-binding specificity of the hSBP/rSBP hybrid

The hSBP/rSBP hybrid, renatured hSBP, and renatured rSBP were separated from each other by electrophoresis as described in Materials and methods, and each was shown to be pure as demonstrated in Figure 5. Binding of E_2 was determined by competitive Scatchard analyses as shown in Figures 6A, B, and C, respectively. Figures 6A and C clearly indicate that renatured hSBP and the hybrid bind E_2 with the same affinity, whereas renatured rSBP does not, as shown in Figure 6B. Note that according to Michaelis-Menten competitive inhibition, the lines in the plots of Figures 6A and B should have converged at the abscissa, because only one class of steroid-binding site per mole SBP dimer exists (Turner et al., 1984). The reason they do not converge in Figures 6A and B is due to a slightly higher SBP concentration in the samples containing E_2 . This is clearly shown in Figure 6B where E_2 does not compete for DHT in renatured rSBP; if the SBP concentration

Fig. 4. Effects of CaCl_2 and DHT on dimerization. Twenty-six microliters of 8 M urea were added to samples of rSBP (15 μg in 10 μl of 10 mM Tris-Cl, 10 mM NaCl, 50% glycerol, pH 7.4) and incubated for 2 hours at 10 °C. Each sample was treated with a dextran-charcoal pellet for 15 minutes at 4 °C. Renaturation was performed by adding 2 ml of 10 mM Tris-Cl, 10% glycerol, pH 7.4, containing either 3 mM EDTA or 5 mM CaCl_2 in the presence or absence of 10 nM DHT. Concentrated samples (20 μl) were electrophoresed on a native slab gel. Lane 1, control rSBP, no urea treatment; lane 2, no additions; lane 3, CaCl_2 addition; lane 4, EDTA plus DHT addition; lane 5, CaCl_2 plus DHT addition; lane 6, EDTA addition.

had been the same, all the data points would have been located on the same line rather than being sequestered into two parallel lines. Table 1 summarizes the equilibrium constants of DHT and E_2 dissociation calculated from all three Scatchard plots, as well as for native human and rabbit SBPs. The K_d values confirm that the hSBP/rSBP hybrid binds both DHT and E_2 with essentially the same affinity as hSBP, whereas rSBP only binds the androgen.

Discussion

The molecular basis of steroid-binding specificity has remained an open question in the field of steroid-binding proteins. For many

Fig. 5. Purity of the hSBP/rSBP hybrid as determined by electrophoresis. Renatured hSBP and rSBP, and the hSBP/rSBP hybrid were purified by preparative electrophoresis and electroelution. Aliquots (2–10 μg in 10 μl) of each purified protein were applied on a native mini gel and electrophoresed in the Protean II system. The gel was stained with Coomassie blue. Lane 1, renatured hSBP; lane 2, hSBP/rSBP hybrid; lane 3, renatured rSBP.

Fig. 6. Determination of equilibrium constants of DHT and E_2 dissociation from (A) renatured hSBP, (B) renatured rSBP, and (C) hSBP/rSBP hybrid by competitive Scatchard analysis, as described in Materials and methods. SBP samples (1–2 nM) were incubated with 0.5–5 nM $[^3\text{H}]\text{DHT}$ in the presence (●) or absence (○) of 400 nM radioinert E_2 .

years, our laboratory has used SBP as a protein model to approach this problem. Because our on-going crystallization efforts have been unproductive thus far, we and others have resorted to indirect approaches such as affinity labeling and site-directed mutagenesis for obtaining structural information on the nature and location of the steroid-binding site. These approaches have led to the identification of the peptide region encompassing K134 and M139 as part of the steroid-binding site (Namkung et al., 1990; Grenot

Table 1. Equilibrium constants of hSBP, rSBP, and hSBP/rSBP hybrid for the dissociation of DHT and E_2

	K_d (nM, 4 °C, pH 7.4)	
	DHT	E_2
Native hSBP	0.76	25.73
Renatured hSBP	0.81	22.36
Native rSBP	1.53	Not measurable ^a
Renatured rSBP	2.52	Not measurable ^a
hSBP/rSBP hybrid	1.31	24.03

^aFor native rSBP, K_d (E_2) is approximately 100-fold higher than K_d (DHT) (Mickelson & Petra, 1978). The competing E_2 concentration used in this experiment was not high enough to obtain competition and estimate its value.

et al., 1992; Sui et al., 1992). Site-directed mutagenesis has also revealed that H235, thought to be located in the steroid-binding site (Khan and Rosner, 1990), is conclusively ruled out from playing any role in steroid-binding (Sui et al., 1992). Because SBP is known to be dimeric with a molar stoichiometry of 1:1 ligand per dimer (Petra, 1979), we proposed a model with one subunit binding the ligand and the other having an undefined role. This proposal was subsequently dismissed when determination of the primary structures of hSBP and rSBP, and other studies, revealed that their subunits were identical (Walsh et al., 1986; Petra et al., 1986a; Griffin et al., 1989). We therefore revised the model with the ligand located at the interface between the subunits (Petra, 1991). The subunits are likely associated “head-to-head” rather than “head-to-tail,” based on the fact that higher aggregates of native hSBP have not been detected in the analytical ultracentrifuge (Petra et al., 1986b), and that visualization of hSBP in the electron microscope reveals individual rod-like shaped structures instead of long fibrils (K. Beck, T. Gruber, C. Ridgway, W. Hughes, L.-M. Sui, & P. Petra, submitted). However, direct biochemical evidence supporting localization of the steroid-binding site at the interface was not obtained because native SBP monomers could not be generated (Petra et al., 1986b); they were only seen as irreversible-denatured monomers in SDS-PAGE. The finding of reversible denaturation in urea therefore provides the first opportunity to examine the proposal. The data presented here indicate that formation of the hSBP/rSBP hybrid requires prior dissociation of the hSBP and rSBP dimers into their respective monomers. Although this process does not require either steroid or calcium, as previously thought (Sui et al., 1995), the steroid could still stabilize the dimer once it is formed (Casali et al., 1990). However, the role of calcium, found in hSBP and rSBP (Ross et al., 1985), remains unknown.

Urea treatment does not cause any effect on the primary structure of SBP. Denatured human and rabbit monomers refold into their native conformations, because they yield dimers with K_d s for DHT and E_2 indistinguishable from those of the native proteins. The peculiar binding specificity of the hSBP/rSBP hybrid, however, is intriguing and strongly suggests that steroid binding takes place at the interface between the subunits. This is based on the fact that the hybrid binds DHT as rSBP does but, unlike rSBP, it binds E_2 as well even though a rSBP monomer is present in the hybrid dimer. The major structural differences between E_2 and DHT, as illustrated in the stereo image of Figure 7 and the kinematic image are (1) flattening and parallel displacement of ring A arising from its aromaticity and a change in the angle joining the planes of

Fig. 7. Stereo view showing the structural differences between 17β -estradiol and DHT. Note the flatness and parallel displacement of ring A of 17β -estradiol and the distinct orientation of its oxygenated group at C3 when the C and D rings of the two steroids are superposed.

rings A and B, (2) loss of the C19 methyl group, and (3) a 45 degree change in the spatial orientation of the oxygenated function on C3. Because rings C and D of DHT and E_2 can be superposed, binding of the two steroids by the hybrid and hSBP can be explained by the presence of a structural motif large enough to enclose both configurations of ring A. The corresponding motif in rSBP is tailored to recognize only the saturated ring A of DHT (and T) with its unique orientation and its protruding C19 methyl group. Furthermore, the structural motif that recognizes ring A of E_2 in the hybrid must be located only in the hSBP subunit because presence of the rSBP subunit does not prevent E_2 from binding. Interestingly, the rSBP monomer still exerts a small effect on the steroid-binding affinity of the hybrid because its K_d of DHT is slightly higher than that of hSBP and closer to that of rSBP (Table 1).

These findings lead us to the following conclusions: (1) "head to head" docking of the two subunits creates an asymmetric steroid-binding site that is located at the interface between the subunits, and (2) only one face of the hSBP dimer contains the structural motif that recognizes the unique orientation of the phenolic flat ring A of E_2 . The structural motif that recognizes the saturated ring A of DHT (and T) with its protruding C19 methyl group is shared by both faces of the dimer because all three proteins bind the androgens equally well. These findings strongly suggest that the steroid-binding site is located at the interface between the subunits. If it were located elsewhere, one would have to return to a negative cooperativity model proposed earlier (Namkung & Petra, 1986) in which steroid binding to one subunit of the dimer induces an allosteric conformation change in the other, preventing a second mole of steroid from binding; this model also suggests a 1:1 stoichiometry. The fact that the hybrid binds both DHT and E_2 , in contrast to rSBP, which does not, makes such a model unlikely that it is more reasonable to explain the direct effect of the associating subunits on the steroid-binding specificity of the dimer by having the ligand bind at the interface. Moreover, it is difficult to rationalize a role for negative cooperativity because the plasma concentrations of sex steroid hormones are much lower than that of SBP, resulting in the latter circulating mostly as a steroid-free dimer. Under such a condition there is little need for negative allosteric regulation.

Finally, observation of an active hSBP/rSBP hybrid indicates that SBP dimerization has been conserved during the molecular evolution of SBP and that the structural elements responsible for dimerization will be homologous in SBPs from other species. Characteristics of the steroid-binding site can now be sought by determining the binding affinities of various DHT, T, and E_2 derivatives to the hybrid in relation to their structures. Eventually, this knowledge will be useful for constructing a model of the SBP molecule in conjunction with X-ray diffraction studies. Resumption of crystallization trials is in progress primarily as a result of the ability to

express active SBP in yeast (L.-M. Sui, C. Ma, I. Woo, & P.H. Petra, submitted). The data presented in this paper also allow us to address the often-asked question of whether the SBP monomer binds steroid. The question can now be regarded as biochemically and physiologically meaningless because the native SBP subunit does not exist on its own, either in plasma or in the purified form. It cannot be detected when the native protein is electrophoresed in native gels or spun in the analytical ultracentrifuge. Steroid-binding activity is only expressed in the dimeric state.

Materials and methods

Materials

5α -Dihydrotestosterone was purchased from Steraloids, Wilton, NH. $[1,2-^3\text{H}]$ DHT (58.4 Ci/mmol) was purchased from New England Nuclear, Wilton, NH. Aquasol-2 was purchased from NEN Research Products. Succinimidyl-6-(biotinamido)hexanoate was purchased from Pierce Chemicals (product #21335, Rockford, IL), and streptavidin, biotinylated alkaline phosphatase, and rabbit anti-goat IgG coupled with alkaline phosphatase were bought from Bio-Rad (Hercules, CA). All other chemicals used were described in previous publications from this laboratory and were reagent grade.

Purification of human and rabbit SBPs

Both SBPs were purified to homogeneity from serum, as previously described for the human protein (Petra & Lewis, 1980; Bordin & Petra, 1980; Griffin et al., 1989), except that DHT was omitted from all buffers used after the affinity chromatography and anti-transferrin immunoadsorption steps. The SBP eluted from the anti-transferrin column (approximately 5 ml) was concentrated and dialyzed overnight at 4 °C against 1 L of 10 mM Tris-Cl, 5 mM CaCl_2 , 10% glycerol (v/v), pH 7.4, and purified by preparative PAGE (Mickelson et al., 1978) but in the absence of DHT. The pure protein was concentrated to 2–4 mg/ml, dialyzed against 20 mM Tris-Cl, 20 mM NaCl, 10 mM CaCl_2 , pH 7.4, and diluted with an equal volume of 100% glycerol. SBP stored at -20°C under these conditions is stable for years without loss of activity. Bound DHT can be removed by treating twice with a charcoal pellet made from four volumes of a dextran-charcoal suspension (500 mg activated charcoal, 50 mg dextran-T70, 100 μL gelatin) for 15 minutes at 25 °C. Protein concentration was determined spectrophotometrically using $\epsilon_{280} = 1.14 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$ (Petra et al., 1986b) and $M_r = 93,400$ (Petra et al., 1986c) for human SBP; and $\epsilon_{280} = 1.27 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$ and M_r for rabbit SBP (Petra et al., 1986b). An average value of $\epsilon_{280} = 1.20 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$ was used to calculate the concentration of the hSBP/rSBP hybrid.

Preparation and purification of polyclonal anti-hSBP

A goat was immunized with pure hSBP and polyclonal antibodies were purified by affinity chromatography on a column of hSBP-agarose as previously described (Bordin & Petra, 1980).

Urea denaturation of hSBP

Aliquots of hSBP (6 μ g in 3 μ l) were added to tubes containing 0.5 M Tris-Cl, pH 6.5, followed by aliquots of an 8 M urea stock solution to yield increasing concentrations of urea (2, 3, 4, and 6 M). The total volume of each reaction was 20 μ l. The samples were incubated at 10 °C for 30 minutes and directly applied to acrylamide gels polymerized in glass tubes (0.5 cm ID $\times$ 14 cm) at the corresponding urea concentrations. The gels contained a 1 cm stacking gel (4% acrylamide, T = 4.2%, C = 1.8%) and a 12 cm separating gel (5% acrylamide, T = 5.2%, C = 3.4%). T represents acrylamide + bisacrylamide expressed as w/v percent, and C represents bisacrylamide expressed as w/w percent of T (Shuster, 1971). Electrophoresis was performed at 120 V for 5 hours at 10 °C until the dye front had migrated to 1 cm from the bottom (Petra et al., 1983). Gels were removed and stained with Coomassie blue. Other samples were rapidly diluted 20-fold with 10 mM Tris-Cl, 5 mM CaCl₂, 10% glycerol, pH 7.4, and concentrated three times to 50 μ l from 450 μ l with the same buffer in a Centricon-10 (Amicon) to remove all the urea prior to electrophoresis.

Purification of renatured hSBP, rSBP, and hSBP/rSBP hybrid

Human SBP (300 μ g in 150 μ l of buffer A: 10 mM Tris-Cl, 10 mM NaCl, 5 mM CaCl₂, 50% glycerol, pH 7.4) was mixed with rSBP (300 μ g in 174 μ l of buffer A) at 10 °C. 972 μ l of 8 M urea kept at 10 °C was added to make the final concentration 6 M urea, and the solution was quickly mixed. The solution was incubated for 2 hours at 10 °C, and quickly added with efficient stirring to 20 ml of ice-cold 10 mM Tris-Cl, 5 mM CaCl₂, 10% glycerol, pH 7.4. The solution was concentrated to 500 μ l in Centricon-10 tubes three times, each time by adding 2 ml of buffer. The final urea concentration was less than 0.02 M. Renatured hSBP, rSBP, and hSBP/rSBP hybrid were purified by electrophoresis on a 2.7 mm thick native polyacrylamide slab gel [3 cm stacking gel (4% acrylamide, T = 4.2%, C = 1.8%) and 11 cm separating gel (5% acrylamide, T = 5.2%, C = 3.4%)]. Power was 120 V for 10 hours at 10 °C. Samples were applied (50 μ l per well). An additional well for Coomassie Blue staining was used to locate the position of each protein. Gel lanes were cut out with a blade and chopped into 0.5 cm pieces. The pure proteins were recovered from the pooled gel slices with the ISCO "Little Blue Tank" electroelutor.

Biotinylation of rSBP and formation of the hSBP/biotinylated rSBP hybrid

Rabbit SBP (600 μ g in 232 μ l of buffer A) was added to 2 ml of 100 mM NaHCO₃, pH 8.5, and passed twice through a Centricon-10 tube to remove Tris-Cl. The solution was concentrated to 100 μ l in the Centricon-10 and to 25 μ l in the speed-vac. NHS-LC-biotin (550 μ g) was dissolved in 250 μ l of ice-cold water, and 25 μ l was added to the 25 μ l SBP solution at 0 °C (15:1 mole reagent per mole SBP). The solution was incubated for 2 hours and reaction was stopped by adding 1 ml of 10 mM Tris-Cl, pH 7.4, and kept overnight. The solution was concentrated 3 times in Centricon-10

to 200 μ l with 10 mM Tris-Cl, pH 7.4, adjusted to 50% glycerol. The final biotinylated rSBP solution (0.88 mg/ml or 9.4×10^{-6} M) was kept at -20 °C. The hSBP/biotinylated rSBP hybrid was prepared as described for the hSBP/rSBP hybrid.

Electroblotting, steroid-binding analyses, and determination of equilibrium constants of steroid dissociation

Electroblotting of native gels (4% stacking gel, T = 4.2%, C = 1.8%; 5% separating gel, T = 5.2%, C = 3.4%) containing 500 ng SBP samples was carried out with the BIO-RAD Modular Mini-Protean II electrophoretic cell according to the manufacturer's instructions. Steroid-binding activity was determined with [³H]DHT and charcoal adsorption (Sui et al., 1992). Detection of radioactivity in electrophoretic tube gels was performed out by first saturating the SBP samples with 10-fold molar excess of [³H]DHT and applying to gels polymerized in the presence of 1–2 nM [³H]DHT (Tabei et al., 1978). After electrophoresis, the gels were removed from the glass tubes, frozen, sliced into 2 mm sections, and counted. The equilibrium constant of E₂ dissociation for hSBP, rSBP, and the hybrid were determined at 4 °C using competitive Scatchard analyses (Mickelson & Petra, 1978). SBP samples (1–2 nM) were incubated with 0.5 to 5 nM [³H]DHT in the presence or absence of 400 nM radioinert E₂ for 30 minutes at 25 °C and 30 minutes at 4 °C. Unbound steroid was removed by treating with dextran-charcoal pellets (prepared from two volumes of 0.5% activated charcoal, 0.05% dextran T-70, 0.1% gelatin) for 2 min at 0 °C. K_d (E₂) was calculated using the equation for competitive inhibition:

$$K_p = K_d(1 + [I]/K_i)$$

where K_d is the equilibrium constant of [³H]DHT dissociation, K_p is K_d in the presence of [I] competitor (400 nM of radioinert E₂), and K_i is the equilibrium constant of E₂ dissociation.

Determination of the effect of DHT and calcium on SBP dimerization

Samples of pure rSBP were denatured in 6 M urea for 2 hours at 11 °C and shaken gently twice at 11 °C for 15 minutes with dextran-charcoal pellets (made from 4 volumes of suspension) to remove DHT. Renaturation by dilution was performed in buffers containing 3 mM EDTA or 5 mM CaCl₂ in the presence or absence of 10 nM DHT. The samples were concentrated and electrophoresed in native gel to estimate the extent of dimerization.

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