Protein Science (1992), *1*, 902-909. Cambridge University Press. Printed in the USA.

Complete enzymatic deglycosylation of native sex steroid-binding protein (SBP or SHBG) of human and rabbit plasma: Effect on the steroid-binding activity

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(RECEIVED January 21, 1992; REVISED MANUSCRIPT RECEIVED February 18, 1992)

Abstract

An enzymatic procedure for the complete removal of the *N*-linked and *O*-linked oligosaccharide side chains of the sex steroid-binding proteins (SBP or SHBG) of human and rabbit plasma under native conditions is described. Deglycosylation was catalyzed by *N*-glycanase, neuraminidase, and *O*-glycanase and was monitored by SDS-PAGE, lectin blotting, and molecular weight analyses by electrospray mass spectrometry. Digestion of rabbit SBP with *N*-glycanase generated a major 39,777-Da protein and two minor ones of 39,389 and 39,545 Da. The molecular weight of the major protein agrees with the molecular weight calculated from the sequence of the sugar-free polypeptide monomer (39,769 Da: Griffin, P.R., Kumar, S., Shabanowitz, J., Charbonneau, H., Namkung, P.C., Walsh, K.A., Hunt, D.F., & Petra, P.H., 1989, *J. Biol. Chem. 264*, 19066-19075), whereas the other two are deglycosylated proteolytic cleavage products lacking the TQR and TQ sequences at the amino-terminus. The N- and *O*-linked side chains of human SBP were removed by sequential digestion with *N*-glycanase and neuraminidase/*O*-glycanase. A 38,771-Da protein was generated, which agrees well with the molecular weight of the sugar-free polypeptide monomer (Walsh, K.A., Titani, K., Kumar, S., Hayes, R., & Petra, P.H., 1986, *Biochemistry 25*, 7584-7590). *N*-deglycosylation of human and rabbit SBP has no effect on the steroid-binding activity, but removal of the *O*-linked side chains of *N*-deglycosylated human SBP results in an apparent 50% loss of steroid-binding activity and an increase in the K_d for the binding of 5α-dihydrotestosterone from 0.3 nM to 0.9 nM. There are no changes in steroid-binding specificity. The apparent loss of activity of *O*-deglycosylated human SBP is probably due to the small changes in the K_d, which could influence the equilibrium concentration of bound SBP when measured under standard assay conditions. We conclude that deglycosylation has very little effect on steroid-binding activity and that the oligosaccharide side chains must serve other functions in the physiology of SBP.

Keywords: deglycosylation; sex hormone-binding globulin; sex steroid-binding protein

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Abbreviations: SBP, plasma sex steroid-binding protein; SHBG, sex-hormone binding globulin; hSBP, human SBP; rSBP, rabbit SBP; ABP, rat androgen-binding protein; *N*-glycanase, peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase; *O*-glycanase, *O*-glycopeptide endo-D-galactosyl-*N*-acetyl-α-galactosamine hydrolase; Con A, concanavalin A; WGA, wheat germ agglutinin; AIA, Jacalin lectin; PNA, peanut agglutinin; RCA, Ricin lectin; DSA, Jimson weed lectin; DHT, 5α-dihydrotestosterone; T, testosterone; E_2, 17β-estradiol; P, progesterone; F, cortisol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; GnHCl, guanidine hydrochloride; PMSF, phenylmethyl sulfonyl fluoride; OP, *O*-phenanthroline; HPLC, high performance liquid chromatography.

The sex steroid-binding proteins of human and rabbit plasma, SBP or SHBG, are homodimeric glycoproteins that specifically bind sex steroid hormones with high affinity (Hammond et al., 1986; Petra et al., 1986; Gershagen et al., 1987; Danzo et al., 1989). A comprehensive review of the biochemistry, molecular biology, and physiology of SBP/SHBG was recently published (Petra, 1991). The human protein specifically binds DHT, T, and E_2, whereas the rabbit protein only binds androgens. Although the function of SBP is not yet clear, the existing data indicate that it controls the metabolic clearance rates

of T and E_2 in plasma (Bardin & Lipsett, 1967; Vermeulen et al., 1969; Petra et al., 1985; Plymate et al., 1990) and perhaps plays a role in their transport across membranes of target cells (Bordin & Petra, 1980; Rosner, 1990; Strel'chyonok & Avvakumov, 1990). The amino acid sequences of human and rabbit SBPs are 79% identical, establishing common ancestry (Walsh et al., 1986; Griffin et al., 1989). The human monomer contains three oligosaccharide side chains (Avvakumov et al., 1983): two *N*-linked at Asn^{351} and Asn^{367} and the other *O*-linked at Thr^{7} (Walsh et al., 1986). The rabbit monomer contains only the two *N*-linked side chains at Asn^{345} and Asn^{361} (Griffin et al., 1989), which are located at the same positions as those of hSBP (rSBP is shorter by six residues at the amino-terminus). The hSBP gene has been cloned (Gershagen et al., 1989; Hammond et al., 1989), and a full-length hSBP/ABP cDNA has been constructed and expressed in BHK-21 and COS-7 cells (Petra, 1991; Hagen et al., 1992). The protein has also been expressed in CHO cells (Bocchinfuso et al., 1991). The steroid-binding site has been localized by affinity-labeling (Grenot et al., 1988; Namkung et al., 1990) and by site-directed mutagenesis (Sui, Cheung, & Petra, in prep.).

The role of the oligosaccharide side chains in the function of SBP is not known. Their discovery came from the work of Van Baelen et al. (1969), who treated crude preparations of hSBP with neuraminidase and compared isoelectrofocusing patterns with untreated protein. They showed that the presence of carbohydrate was partly responsible for the microheterogeneity of the protein and that removal of terminal sialic acid residues had no effect on the steroid-binding activity. Although data from other laboratories were consistent with these observations (Suzuki & Sinohara, 1979; Cheng et al., 1985), complete deglycosylation under native conditions was not achieved because the enzymes used could not catalyze the hydrolysis of oligosaccharide side chains linked to asparaginyl and seryl/threonyl residues. Therefore, despite recent claims, the existing data do not support any conclusion on whether or not the oligosaccharide side chains play a role in the steroid-binding activity of SBP. Because the side chains are located at the carboxy- and amino-termini of each monomeric polypeptide chain, we reasoned that they might be well exposed to solvent and accessible to attack by glycopeptide amidases and hydrolases under conditions mild enough to maintain the native conformation. In this report we describe a procedure for the complete removal of the oligosaccharide side chains under native conditions and discuss the consequences of such structural changes on the steroid-binding activity of both human and rabbit SBP.

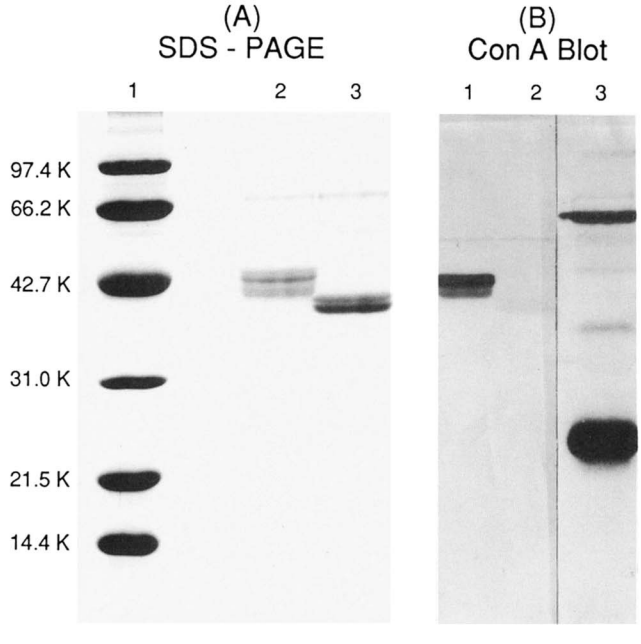

Fig. 1. SDS-PAGE and Con A blot of native and deglycosylated rSBP. Electrophoresis of 5 μg protein in 0.1% SDS (0.7-mm slab gels, 10% running, 5% stacking, 3% methylenebisacrylamide) as described by Petra et al. (1983, 1986). **A:** Coomassie blue stain; lane 1, 2.5 μL of Bio-Rad low molecular weight markers; lane 2, native SBP; lane 3, *N*-glycanase-digested SBP. **B:** Con A blot: lane 1, native SBP; lane 2, *N*-glycanase-digested SBP; lane 3, transferrin and ribonuclease B markers (Genzyme).

Results

Deglycosylation of rSBP

Complete removal of the two *N*-linked oligosaccharide side chains of rSBP was achieved with recombinant *N*-glycanase. The SDS-PAGE patterns of native rSBP before (lane 2) and after *N*-glycanase digestion (lane 3) are shown in Figure 1A. All the native bands disappeared, and deglycosylated rSBP was resolved into a major band of about 39 kDa and a minor band of slightly higher mass. The estimated molecular weight of 39 kDa for the major band agrees with the sugar-free subunit polypeptide molecular weight of 39,769 of rSBP as determined from the amino acid sequence (Griffin et al., 1989). Thus, *N*-glycanase digestion of rSBP (molecular weight of fully glycosylated rSBP = 43,702; Griffin et al., 1989) produces a protein having an estimated mass loss of about 5 kDa, which is consistent with the removal of two *N*-linked complex high mannose biantennary oligosaccharide side chains per subunit (5.2 kDa).

Complete removal of the *N*-linked side chains was confirmed by lectin blotting and mass spectrometry. Con A blots before (lane 1) and after *N*-glycanase treatment (lane 2) are shown in Figure 1B. Note that the same amount of SBP (4–5 μg) was applied to all lanes, indicating that the lack of staining in lane 2 of Figure 1B is not due to insufficient protein. Absence of *O*-linked side chains in rSBP was confirmed by negative staining with

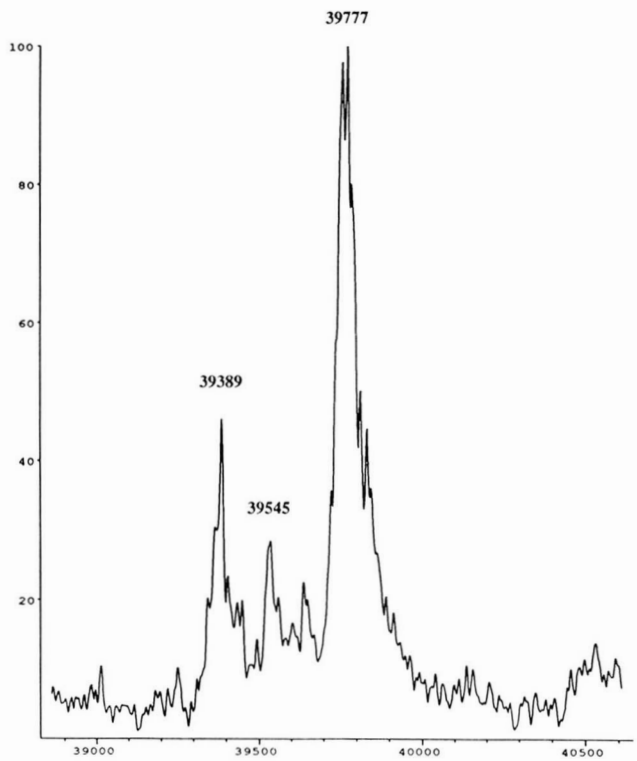

Fig. 2. Derived spectrum of an electrospray mass spectrum of deglycosylated rSBP. The major peak of 39,777 Da corresponds to fully deglycosylated SBP. The native SBP monomer was found to have a mass of 44,200 (see Table 1, spectrum not shown). The 39,389-Da and 39,545-Da peaks correspond to deglycosylated monomer lacking the amino-terminal sequence TQR (mass = 404) and TQ (mass = 247), respectively.

RCA and PNA, lectins specific for *O*-linked side chains (data not shown).

The mass spectrometric data are shown in Figure 2 and Table 1. Treatment with *N*-glycanase generates a major

Table 1. *Molecular masses of native and deglycosylated monomeric subunits of rSBP and hSBP derived from electrospray mass spectra (all values in daltons)*

	rSBP	hSBP
Native monomers	44,200	44,482 ± 44[a]
Deglycosylated monomers		
Full-length monomer	39,777	38,771 ± 39[a]
Monomer lacking amino-terminus TQR	39,389	–
Monomer lacking amino-terminus TQ	39,545	–

[a] These values were recorded with matrix-assisted laser desorption-time-of-flight mass spectrometry.

rSBP species of 39,777 Da and two minor ones of 39,389 and 39,545 Da. The mass of the major peak is in excellent agreement with the amino acid sequence (39,769) and the SDS-PAGE data presented above, whereas the mass of the other two peaks corresponds to proteolytic cleavage products composed of the polypeptide monomer lacking the TQR and TQ sequences at the amino-terminus. These were previously detected during determination of the amino acid sequence (Griffin et al., 1989).

Deglycosylation of hSBP

Complete removal of the *N*- and *O*-linked oligosaccharide chains from hSBP was achieved by digesting with recombinant *N*-glycanase followed by a mixture of neuraminidase and *O*-glycanase. The use of neuraminidase is required because sialic acid residues inhibit *O*-glycanase. The SDS-PAGE patterns of native hSBP (lane 2), hSBP digested with *N*-glycanase (lane 3), and *N*-deglycosylated-hSBP digested with neuraminidase/*O*-glycanase (lane 4) are shown in Figure 3A. All of the native SBP bands dis-

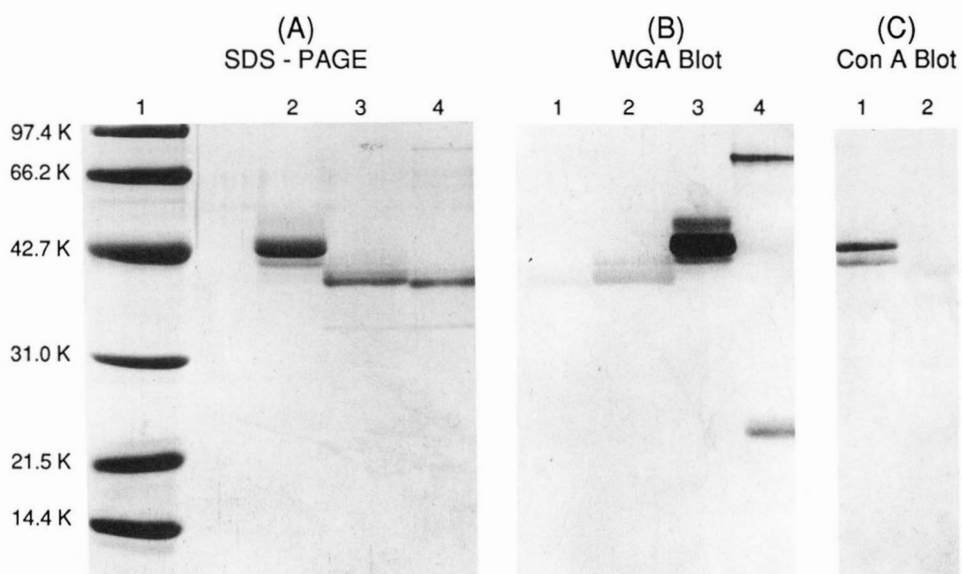

Fig. 3. SDS-PAGE and Con A blot of 5 μg of native and deglycosylated hSBP. Same conditions as in Figure 1. **A:** Coomassie blue stain: lane 1, 2.5 μL of Bio-Rad low molecular weight markers; lane 2, native SBP; lane 3, *N*-glycanase-digested SBP; lane 4, *N*-glycanase-digested SBP treated with neuraminidase/*O*-glycanase. **B:** WGA blot: lane 1, glycanase-digested SBP treated with neuraminidase/*O*-glycanase; lane 2, *N*-glycanase-digested SBP; lane 3, native SBP; lane 4, transferrin and ribonuclease B markers (Genzyme). **C:** Con A blot: lane 1, native SBP; lane 2, *N*-glycanase-digested SBP.

appeared, and the protein is resolved into a new band migrating at about 39 kDa and a trace band at slightly higher mass. The new molecular weight agrees with the sugar-free subunit polypeptide molecular weight of 40,499 determined from the hSBP amino acid sequence (Walsh et al., 1986). Removal of the *O*-linked side chain can be seen in the SDS-PAGE pattern of Figure 3A, where the stained band in lane 4 migrates slightly faster than in lane 3.

Complete deglycosylation was confirmed by lectin blotting and mass spectrometry. A blot of a duplicate SDS gel was probed with WGA (Fig. 3B) and Con A (Fig. 3C). The former lectin recognizes both β-*N*-acetylglucosamine and sialic acid residues but not branched mannoses, whereas the latter is specific for both branched mannoses and β-*N*-acetylglucosamine residues but not for sialic acid residues. Digestion with *N*-glycanase eliminated Con A staining (lane 2, Fig. 3C), indicating removal of all *N*-linked side chains. Positive staining with WGA indicates that the *O*-linked side chain is still bound to Thr^7 due to the presence of the sialic acid residues on that side chain (lane 2, Fig. 3B). When *N*-deglycosylated SBP was further digested with neuraminidase and *O*-glycanase, WGA staining disappeared, demonstrating that the sialic acid residues were removed along with the *O*-linked side chain (lane 1, Fig. 3B). Blots after neuraminidase/*O*-glycanase probed with RCA, specific for terminal β-galactose and β-galactosamine, and with PNA, specific for D-Gal-β(1,3)GalNAc, were also negative (data not shown). These data combined with those of Figure 3A indicate that the *O*-linked side chain was enzymatically removed from hSBP.

Mass spectrometric analyses were also carried out to corroborate the SDS-PAGE and lectin blot data. Table 1 shows that sequential digestion with *N*-glycanase and neuraminidase/*O*-glycanase produced a 38,771-Da molecular species with a mass loss of 5,711 Da when compared to the native monomer (44,482 Da). Thus, complete deglycosylation resulted in an estimated mass loss of about 6 kDa, which agrees with the removal of two *N*-linked (5.2 kDa) and one *O*-linked (1 kDa) oligosaccharide side chains per subunit, consistent with the data of Avvakumov et al. (1983). The results in Table 1 and Figures 1–3 are therefore consistent with the removal of the oligosaccharide side chains of human and rabbit SBP. To be noted is that no remaining native rSBP and hSBP was found by either SDS-PAGE or mass spectrometry following enzyme digestion, indicating further that deglycosylation was complete.

Steroid-binding activity, Scatchard analyses, and steroid-binding specificity of deglycosylated hSBP and rSBP

The steroid-binding properties of deglycosylated SBPs are shown in Tables 2–4 and in Figure 4. Removal of the *N*-linked side chains of human and rabbit SBP resulted

Table 2. *DHT binding activity of native and deglycosylated SBP*[a]

	Human		Rabbit	
	Total activity (nM)	% Activity remaining	Total activity (nM)	% Activity remaining
Control	25.2	100	29.5	100
N-Deglycosylation	23.6	94	25.8	87
O-Deglycosylation	12.1	48	–	–

[a] The values represent the average of two to three different experiments. The activity was measured with the standard filter assay as described in the Materials and methods. The SBP protein concentrations were normalized before assay. Controls were carried through the same time incubations and dialyses.

Table 3. *Equilibrium constants of native and deglycosylated hSBP for the dissociation of DHT (4 °C, pH 7.4)*

Experiment	Fully deglycosylated (nM)	Native (nM)
1[a]	0.83	0.36[b]
2	0.93	0.40[c]
3	0.96	0.24[d]

[a] The Scatchard plot is shown in Figure 4. The average value of the K_d based on all three determinations is 0.91 nM.
[b] Mickelson and Petra (1974).
[c] Renoir et al. (1980).
[d] Shanbhag and Sodergard (1986).

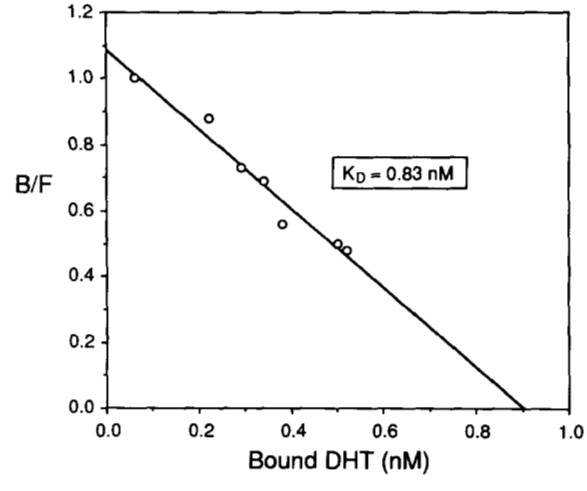

Fig. 4. Determination of the equilibrium constant for the dissociation of DHT from fully deglycosylated hSBP. The correlation coefficient for the linear regression shown, $y = -(1.20\ nM^{-1})x + 1.09$, was $r = 0.98$. The K_d, 0.83 nM, is the reciprocal of the slope ($1/1.20\ nM^{-1}$). Two other Scatchard analyses were carried out, yielding K_d's of 0.93 nM and 0.96 nM; the correlation coefficients were 0.93 and 0.99, respectively. The average value of the K_d is 0.91 nM (Table 3).

Table 4. *Relative steroid-binding specificity of deglycosylated SBP (%)*

	hSBP		rSBP	
Steroid	Deglycosylated	Native	Deglycosylated	Native[a]
5α-Dihydrotestosterone	100	100	100	100
Testosterone	56	58	71	44[b]
17β-Estradiol	12	13	8	5
Progesterone	0	3	0	1
Cortisol	0	8	0	1

[a] Mahoudeau and Corvol (1973); Rosner and Darmstadt (1973).
[b] Average of reported values by Mahoudeau and Corvol (1973) and Rosner and Darmstadt (1973).

in very little loss of steroid-binding activity, if any. The slight loss could arise from a change in the SBP molar extinction coefficient used to normalize the concentrations of deglycosylated SBP after dialysis. In contrast, a reproducible 50% loss was found after the removal of the *O*-linked side chains of hSBP. The Scatchard plot of Figure 4 and data in Table 3 reveal a K_d value of 0.91 nM (averaged from three different determinations) for DHT binding to fully deglycosylated hSBP, which, when compared to the K_d of 0.3 nM for the native protein (Table 3), is only three times that value as well as that of recombinant SBP (Hagen et al., 1992). Table 4 indicates that the relative steroid-binding specificity of fully deglycosylated human and rabbit SBPs, DHT > T > E_2 > P and F, is the same as for native hSBP.

Discussion

Deglycosylation of glycoproteins in their native states is necessary for understanding the role of oligosaccharide side chains in structure/function relationships. Complete carbohydrate removal is desirable but often difficult because secondary and tertiary structure of the polypeptide chain may obstruct or prevent the enzymatic action of glycosidases. In the case of human and rabbit SBPs, complete deglycosylation under native conditions was particularly challenging because the proteins are homodimers and contain at least 12 different isoforms resulting from variable glycosylation (Kotite & Musto, 1982; Petra et al., 1983; Gershagen et al., 1987; Danzo et al., 1989). However, because the oligosaccharide chains of these proteins are located at the extremities of the molecule (Walsh et al., 1986; Griffin et al., 1989), they could be significantly exposed to solvent thereby facilitating their removal under conditions mild enough to maintain the active protein conformation. Deglycosylation of hSBP was previously attempted in several laboratories with limited success (including our unpublished results). Cheng et al. (1985) have shown that a large portion of the carbohydrate can be removed under native conditions using a combination of different glycosidases. Danzo et al. (1989) were able to remove all the oligosaccharide side chains using the enzymes employed in the work presented here, but the conditions used (100 °C, 0.5% SDS, 0.1 M β-mercaptoethanol) were too harsh to maintain the steroid-binding activity. Although these procedures could not be used for our purpose here, this latter finding nevertheless indicated that complete deglycosylation was feasible.

There are two commercially available enzymes that catalyze the complete removal of oligosaccharide side chains from glycoproteins. The first, *N*-glycanase (Genzyme Corp.), cleaves off *N*-linked side chains at asparagine residues, converting them to aspartyl residues (Tarentino et al., 1985). The second enzyme, *O*-glycanase (Genzyme Corp.), cleaves off *O*-linked side chains at threonine and serine residues (Umemoto et al., 1977). Because rSBP does not contain *O*-linked side chains, we first attempted to deglycosylate it with *N*-glycanase under native conditions and found that only the oligosaccharide chain attached to Asn^{367} was preferentially removed (Moore et al., 1991). However, as described here, when the experiment was repeated with recombinant *N*-glycanase, both *N*-linked chains were cleaved off. We cannot explain the "superactivity" of the recombinant enzyme, but it could arise from a higher state of purity and/or from the absence of inhibitors. The same results were obtained with hSBP when the wild-type enzyme was replaced with the recombinant enzyme.

Removal of all four *N*-linked side chains from dimeric hSBP and rSBP has very little effect, if any, on their steroid-binding activities. An apparent reproducible loss of 50% activity measured with the standard filter assay was observed when the *O*-linked side chains were removed from *N*-deglycosylated hSBP. The K_d of DHT binding to fully deglycosylated hSBP, however, is only three times the value of the native protein, which is an extremely small difference considering the absolute values of these constants (nanomolar range). In addition, both native and fully deglycosylated proteins have the same relative steroid-binding specificities. We therefore conclude that *O*-deglycosylation does not significantly alter the steroid-binding properties of hSBP; the observed 50% loss of

activity is probably due to small changes in the K_d that could influence the equilibrium concentration of bound SBP when measured under standard assay conditions. Nevertheless, this slight increase in the K_d could point to a subtle structural change in either the overall conformation of the protein or the steroid-binding site. If the amino-terminus of one of the subunits with its *O*-linked chain were to fold in the vicinity of the steroid-binding site and contribute to its stability, removal of the side chain might disrupt the local conformation to some extent and affect the activity of the protein. On the other hand, loss of activity could result from a global rather than local conformation change. Interestingly, the K_d for DHT binding to native rSBP reported to be 0.9 nM at 4 °C (Mickelson & Petra, 1978; Petra et al., 1986) is comparable to that obtained here for fully deglycosylated hSBP (Table 3). Because this value is three times that reported for native and recombinant hSBP, presence of the *O*-linked side chain on Thr^7 may increase slightly the steroid-binding affinity of SBP through either one of the two mechanisms proposed. Perhaps glycosylation at Thr^7 represents a selected event in the evolution of primate SBPs having slightly higher steroid-binding affinities than those of lower species.

We therefore conclude that the oligosaccharide side chains must serve more important functions in the physiology of SBP than binding steroids. We speculate on three possibilities involving intracellular trafficking and extracellular targeting. First, the bound sugars may act in the secretion of SBP from liver (or other tissues) into plasma. The side chains may also function indirectly in sex steroid hormone secretion through the trafficking of SBP–steroid complexes from intracellular compartments to plasma. Some years ago we had proposed that intracellular SBP could play a role in the secretion of sex steroid hormones from cells that synthesize them (Bordin & Petra, 1980). Because full-length SBP cDNAs have now been expressed in mammalian cells (Bocchinfuso et al., 1991; Hagen et al., 1992), this proposal can be tested by transfecting SBP mutagenic at Asn^{351}, Asn^{367}, and Thr^7, and looking for a lack of SBP secretion. Second, the oligosaccharide side chains may function by maintaining a steady-state level of the protein in plasma, thereby regulating its metabolic clearance rate. This hypothesis is supported by the data of Suzuki and Sinohara (1979) who found that asialo-hSBP is taken up faster by rat liver cells than native SBP, suggesting that removal of sialic acid residues increases SBP clearance. That proposal can also be tested by determining the half-life of deglycosylated hSBP in monkey plasma and comparing it to native hSBP (Namkung et al., 1989). Third, the oligosaccharide side chains may function by targeting SBP to its membrane receptor. Availability of deglycosylated SBP allows testing of the idea directly by carrying out membrane-binding analyses. This approach circumvents the need for using recombinant SBP mutated at oligosaccharide attachment sites, which may be difficult to isolate if the oligosaccharide side chains are required for secretion of the protein into the medium of the transfected cells. Although performance and interpretation of all these experiments may be problematic, the data collected should nevertheless provide a better understanding of the function of the oligosaccharide side chains in the biological activity and physiological role of SBP.

Materials and methods

Materials

Human pregnancy serum was obtained from Madigan Army Hospital, Tacoma, Washington, and rabbit serum from Pel-Freez Biologicals. DHT was purchased from Steraloids and [1,2-^{3}H]DHT (specific activity 2,160.8 GBq/mmol) from New England Nuclear. DEAE-cellulose filter paper discs (DE-81, 2.3 cm diameter) were purchased from Reeve Angel & Co. Aquasol-2 was purchased from NEN Research Products. Natural and recombinant *N*-glycanase (EC 3.5.1.52), albumin-free *O*-glycanase (EC 3.2.1.97), and the following biotinylated lectins—Con A (from *Canavalia ensiformis*), WGA (from *Triticum vulgaris*), DSA (from *Datura stramonium*), and RCA (from *Ricinus communis*)—were all purchased from Genzyme. Avidin–alkaline phosphatase and biotinylated PNA (from *Arachis hypogaea*) were purchased from E.Y Laboratories. *Clostridium perfringens* neuraminidase (type X) was obtained from Sigma. All other chemicals used were described in previous publications from this laboratory and were reagent grade.

Purification of human and rabbit SBP

Both SBPs were purified to homogeneity from human and rabbit sera as previously described (Bordin & Petra, 1980; Petra & Lewis, 1980; Griffin et al., 1989) with some modifications (Namkung et al., 1990). The concentrations of SBP were determined spectrophotometrically using $\epsilon_{280} = 1.14 \times 10^5\ cm^{-1}\ M^{-1}$ for hSBP and $\epsilon_{280} = 1.27 \times 10^5\ cm^{-1}\ M^{-1}$ for rSBP (Petra et al., 1986).

Deglycosylation of rSBP

Five units of *N*-glycanase (20 μL) were added to 500 μg protein in 200 μL of 0.1 M Tris-Cl, 6 mM PMSF, 6 mM OP, 0.01% NaN_3, pH 8.5. The reaction tube was capped and placed at 37 °C for 72 h. The solution was dialyzed overnight at 25 °C against 200 mL of 10 mM Tris-Cl, 10 mM NaCl, pH 7.4. Samples were removed for SBP assays, lectin blotting, molecular weight determination by SDS-PAGE and mass spectrometry, and UV spectra for protein concentration measurement.

Deglycosylation of hSBP

Five hundred micrograms of hSBP was dialyzed overnight at 4 °C against 0.1 M Tris-acetate, pH 8.0, and di-

gested with *N*-glycanase as described above. The solution was then dialyzed overnight at 25 °C against 200 mL of 0.1 M Tris-acetate, pH 8.0, and samples were removed for analyses. The remaining *N*-deglycosylated SBP was first digested with 0.004 units of neuraminidase per microgram of SBP for 7 h at 37 °C and then with 0.13 milliunits of *O*-glycanase per microgram of SBP for an additional 24 h at 37 °C. The solution was dialyzed for 24 h at 4 °C against 200 mL of 10 mM Tris-Cl, 10 mM NaCl, pH 7.4, and samples were removed for analyses.

SBP assays and ELISA

The DEAE-cellulose filter assay previously described (Mickelson & Petra, 1974; Schiller & Petra, 1975) was used with modification for pure SBP (Namkung et al., 1989, 1990). The standard assay was carried out in the presence of 0.04% BSA with three times molar excess of [^{3}H]DHT over SBP, the filters were counted in an Omnifluor/toluene cocktail. Competition studies with other steroids were carried out on 150-μL samples containing 20 nM native or deglycosylated SBP with 20 nM [^{3}H]DHT and 5- or 10-fold molar excess of radioinert DHT, T, E_2, P, or F. ELISA was performed as previously published (Bordin et al., 1982). Polyclonal monospecific hSBP antibodies were prepared from goat anti-plasma using protein G–agarose and hSBP–agarose affinity chromatographies. The pure antibodies were conjugated to alkaline phosphatase (Sigma type VII-NT) with glutaraldehyde, and SBP was determined using *p*-nitrophenyl phosphate.

Gel electrophoresis and lectin blots

SDS-PAGE was carried out as published (Petra et al., 1983, 1986). Proteins in SDS slab gels were transferred to nitrocellulose membranes with the Transblot apparatus (Bio-Rad) according to instructions by the manufacturer. The blots were fixed for 10 min in 10 mL of 10% acetic acid (v/v) and 25% isopropanol (v/v); rinsed in 10 mL of blocking buffer (2% gelatin in 0.5 M NaCl, 0.02 M Tris-Cl, 0.1 mM $CaCl_2$, $MgCl_2$, $MnCl_2$, pH 7.4); and blocked in 10 mL of blocking buffer for 30 min at 25 °C (or overnight at 4 °C). Each blot was then incubated for 1 h at 25 °C with 10 mL blocking buffer containing one of the following biotinylated lectins: Con A (14 μg), WGA (80 μg), RCA (80 μg), DSA (80 μg), PNA (80 μg), or AIA (80 μg). After rinsing three times with 10 mL wash buffer (0.1% Tween 20, 0.5 M NaCl, 0.02 M Tris-Cl, 0.1 mM $CaCl_2$, $MgCl_2$, $MnCl_2$, pH 7.4), the blot was incubated for 1 h with 10 mL of wash buffer containing 20 μL of avidin–alkaline phosphatase. After rinsing with 10 mL of staining buffer (0.1 M Tris-Cl, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5), the blot was stained in 10 mL of staining buffer containing 66 μL of nitro blue tetrazolium solution (50 mg/mL, Promega) and 33 μL of 5-bromo-4-chloro-3-indolylphosphate (50 mg/mL, Promega).

HPLC

SBP was fractionated by reverse-phase capillary HPLC with a gradient of 0 (0.085% trifluoroacetic acid in water) to 100% acetonitrile containing 0.1% trifluoroacetic acid using an Applied Biosystems 140 A solvent delivery system. The HPLC pump was operated at 100 μL/min, and the flow was split prior to the sample injection loop (0.5 μL) at a ratio of 50 to 1. Capillary columns were 320 μm internal diameter × 15 cm and were packed with 5-μm C18 particles. Eluent was monitored at 220 nm using a Spectroflow 783 UV detector equipped with an LC Packings U-shaped capillary flow cell (path length = 7 mm). Signals from the UV detector were fed directly to the DECstation 2100 of the mass spectrometer. Eluent exiting the detector was directed into the electrospray ion source using 50-μm capillary tubing.

Mass spectrometry

Mass spectrometry was performed on native and fully deglycosylated SBP fractionated by reverse-phase capillary HPLC and fed directly to a Finnigan MAT TSQ-700 triple quadrupole mass spectrometer. Mass spectra were recorded on the triple quadrupole mass spectrometer equipped with a 20-keV conversion dynode, DECstation 2100, and an electrospray ion source. The design of the electrospray source has been described in detail elsewhere (Whitehouse et al., 1985; Griffin et al., 1991). The molecular weights of native and deglycosylated SBP were recorded by scanning the third quadrupole at a rate of 400 atomic mass units/s over a mass of 600–1,200 throughout the HPLC gradient.

Acknowledgments

We thank Pearl C. Namkung for her contributions to the purification of SBP and for her helpful suggestions. This research was supported by the following grants: NICHD-13956 (P.H.P.), contract 8809710 for the Science and Technology Centers of NSF (P.R.G. and J.R.Y.), and Finnigan MAT (P.R.G. and J.R.Y.).

References

Avvakumov, G.V., Matveentseva, I.V., Akhrem, L.V., Strel'chyonok, O.A., & Akhrem, A.A. (1983). Study of the carbohydrate moiety of human serum sex hormone-binding globulin. *Biochim. Biophys. Acta 760*, 104–110.

Bardin, C.W. & Lipsett, M.B. (1967). Testosterone and androstenedione blood production rates in normal women and women with idiopathic hirsutism or polycystic ovaries. *J. Clin. Invest. 46*, 891–902.

Bocchinfuso, W.P., Warmels-Rodenhiser, S., & Hammond, G.L. (1991). Expression and differential glycosylation of human sex hormone-binding globulin by mammalian cell lines. *Mol. Endocrinol. 5*, 1723–1729.

Bordin, S. & Petra, P.H. (1980). Immunocytochemical localization of the plasma sex steroid-binding protein (SBP) in tissues of the adult male monkey, *Macaca nemestrina. Proc. Natl. Acad. Sci. USA 77*, 5678–5682.

Bordin, S., Torres, R., & Petra, P.H. (1982). An enzyme immunoassay (ELISA) for the sex steroid-binding protein (SBP) of human plasma. *J. Steroid Biochem. 17*, 453–457.

Cheng, C.Y., Musto, N.A., Gunsalus, G.L., & Bardin, C.W. (1985). The role of the carbohydrate moiety on the size heterogeneity and immunologic determinants of human testosterone–estradiol-binding globulin. *J. Steroid Biochem. 22*, 127–134.

Danzo, B.J., Bell, B.W., & Black, J.H. (1989). Human testosterone-binding globulin is a dimer composed of two identical protomers that are differentially glycosylated. *Endocrinology 124*, 2809–2817.

Gershagen, S., Henningsson, K., & Fernlund, P. (1987). Subunits of human sex hormone binding globulin. Interindividual variation in size. *J. Biol. Chem. 262*, 8430–8437.

Gershagen, S., Lundwall, A., & Fernlund, P. (1989). Characterization of the human sex hormone binding globulin (SHBG) gene and demonstration of two transcripts in both liver and testis. *Nucleic Acids Res. 17*, 9245–9258.

Grenot, C., Montard, A., Blachere, T., Mappus, E., & Cuilleron, C.V. (1988). Identification d'un site de photomarquage de la proteine plasmatique de liaison de la testosterone et de l'estradiol (SBP) par l'hydroxy-17β oxo-3 androstadiene-4,6 tritie. *C. R. Acad. Sci. Paris, Serie III 307*, 391–396.

Griffin, P.R., Coffman, J.A., Hood, L.E., & Yates J.R., III. (1991). Structural analysis of proteins by capillary HPLC electrospray tandem mass spectrometry. *Int. J. Mass Spectrom. Ion Processes 111*, 131–149.

Griffin, P.R., Kumar, S., Shabanowitz, J., Charbonneau, H., Namkung, P.C., Walsh, K.A., Hunt, D.F., & Petra, P.H. (1989). The amino acid sequence of the sex steroid-binding protein of rabbit serum. *J. Biol. Chem. 264*, 19066–19075.

Hagen, F.S., Arguelles, C., Sui, L.M., Zhang, W., Seidel, P.R., Conroy, S.C., & Petra, P.H. (1992). Mammalian expression of the human sex steroid-binding protein of plasma (SBP or SHBG) and Testis (ABP). Characterization of the recombinant protein. *FEBS Lett. 299*, 23–27.

Hammond, G.L., Robinson, P.A., Sugino, H., Ward, D.N., & Finne, J. (1986). Physicochemical characteristics of human sex hormone binding globulin: Evidence for two identical subunits. *J. Steroid Biochem. 24*, 815–824.

Hammond, G.L., Underhill, D.A., Rykse, H.M., & Smith, C.L. (1989). The human sex hormone-binding globulin gene contains exons for androgen-binding protein and two other testicular messenger RNAs. *Mol. Endocrinol. 3*, 1869–1876.

Kotite, N.J. & Musto, N.A. (1982). Subunit structure of rabbit testosterone estradiol-binding globulin. *J. Biol. Chem. 257*, 5118–5123.

Mahoudeau, J.A. & Corvol, P. (1973). Rabbit testosterone-binding globulin. I. Physico-chemical properties. *Endocrinology 92*, 1113–1119.

Mickelson, K.E. & Petra, P.H. (1974). A filter assay for the sex steroid-binding protein (SBP) of human serum. *FEBS Lett. 44*, 34–38.

Mickelson, K.E. & Petra, P.H. (1978). Purification and physico-chemical characterization of the sex steroid-binding protein of rabbit serum. Comparison with the human protein. *J. Biol. Chem. 253*, 5293–5298.

Moore, K.H., Plymate, S.R., Griffin, P.R., & Petra, P.H. (1991). Enzymatic deglycosylation of rabbit SBP and its effect on steroid binding. 73rd Annual Meeting of the Endocrine Society, Washington, D.C. [Abstr.]

Namkung, P.C., Kumar, S., Walsh, K.A., & Petra, P.H. (1990). Identification of lysine-134 in the steroid binding site of the sex steroid binding protein of human plasma. *J. Biol. Chem. 265*, 18345–18350.

Namkung, P.C., Stanczyk, F.Z., Cook, M.J., Novy, M.J., & Petra, P.H. (1989). Half-life of plasma sex steroid-binding protein (SBP) in the primate. *J. Steroid Biochem. 32*, 675–680.

Petra, P.H. (1991). The plasma sex steroid binding protein (SBP or SHBG). A critical review of recent developments on the structure, molecular biology, and function. *J. Steroid Biochem. 40*, 735–753.

Petra, P.H. & Lewis, J. (1980). Modification in the purification of the sex steroid-binding protein of human serum by affinity chromatography. *Anal. Biochem. 105*, 165–169.

Petra, P.H., Namkung, P.C., Senear, D.F., McCrae, D.A., Rousslang, K.W., Teller, D.C., & Ross, J.B.A. (1986). Molecular characterization of the sex steroid-binding protein (SBP) of plasma. Re-examination of rabbit SBP and comparison with the human, macaque, and baboon proteins. *J. Steroid Biochem. 25*, 191–200.

Petra, P.H., Stanczyk, F.Z., Namkung, P.C., Fritz, M.A., & Novy, M.J. (1985). Direct influence of the sex steroid-binding protein (SBP) of plasma on the metabolic clearance rate of testosterone. *J. Steroid Biochem. 22*, 739–746.

Petra, P.H., Stanczyk, F.Z., Senear, D.F., Namkung, P.C., Novy, M.J., Ross, J.B.A., Turner, E., & Brown, J.A. (1983). Current status of the molecular structure and function of the plasma sex steroid-binding protein (SBP). *J. Steroid Biochem. 19*, 699–706.

Plymate, S.R., Namkung, P.C., Matej, L.A., & Petra, P.H. (1990). Direct effect of plasma sex hormone binding globulin (SHBG) on the metabolic clearance rate of 17β-estradiol in the primate. *J. Steroid Biochem. 36*, 311–317 .

Renoir, J.-M., Mercier-Bodard, C., & Baulieu, E.-E. (1980). Hormonal and immunological aspects of the phylogeny of sex steroid binding protein. *Proc. Natl. Acad. Sci. USA 77*, 4578–4582.

Rosner, W. (1990). The functions of corticosteroid-binding globulin and sex hormone-binding globulin: Recent advances. *Endocrinol. Rev. 11*, 80–91.

Rosner, W. & Darmstadt, R. (1973). Demonstration and partial characterization of a rabbit serum protein which binds testosterone and dihydrotestosterone. *Endocrinology* 92, 1700–1707.

Schiller, H. & Petra, P.H. (1975). A filter assay for the corticosteroid-binding globulin (CBG) of human serum. *J. Steroid Biochem. 7*, 55–59.

Shanbhag, V.P. & Sodergard, R. (1986). The temperature dependence of the binding of 5α-dihydrotestosterone, testosterone, and estradiol to the sex hormone binding globulin (SHBG) of human plasma. *J. Steroid Biochem. 24*, 549–555.

Strel'chyonok, O.A. & Avvakumov, G.V. (1990). Specific steroid-binding glycoproteins of human blood plasma: Novel data on their structure and function. *J. Steroid Biochem. 35*, 519–534.

Suzuki, Y. & Sinohara, H. (1979). Hepatic uptake of desialylated testosterone–estradiol-binding globulin in the rat. *Acta Endocrinol. 90*, 669–679.

Tarentino, A.L., Gomez, C.M., & Plummer, T.H. (1985). Deglycosylation of asparagine-linked glycans by peptide:N-glycosydase F. *Biochemistry 24*, 4665–4671.

Umemoto, J., Bhavanandan, V.P., & Davidson, E.A. (1977). Purification and properties of an endo-α-*N*-acetyl-D-galactosaminidase from *Diplococcus pneumoniae. J. Biol. Chem. 252*, 8609–8614.

Van Baelen, H., Heyns, W., & DeMoor, P. (1969). Microheterogeneity of the testosterone-binding globulin of human pregnancy serum demonstrated by isoelectric focusing. *Ann. Endocrinol. 30*, 199.

Vermeulen, A.L., Verdonck, L., Van der Straeten, M., & Orie, M. (1969). Capacity of the testosterone binding globulin in human plasma and influence of specific binding of testosterone on its metabolic clearance rate. *J. Clin. Endocrinol. Metab. 29*, 1470–1480.

Walsh, K.A., Titani, K., Kumar, S., Hayes, R., & Petra, P.H. (1986). Amino acid sequence of the sex steroid-binding protein (SBP) of human blood plasma. *Biochemistry 25*, 7584–7590.

Whitehouse, C.M., Dreyer, R.N., Yamashita, M., & Fenn, J.B. (1985). Electrospray interface for liquid chromatographs and mass spectrometers. *Anal. Chem. 57*, 675–677.