Purification and Characterization of the Sex Steroid Binding Protein from Macaque Serum. Comparison with the Human Protein†

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ABSTRACT: The sex steroid binding protein (SBP) of *Macaca mulatta* and *Macaca nemestrina* sera has been purified to homogeneity and chemically characterized. The native protein is a glycoprotein having a molecular weight of approximately 88 000 and is composed of two similar subunits of molecular weight 47 000 as estimated by sodium dodecyl sulfate gel electrophoresis. One molecule of 5α-dihydrotestosterone is bound per dimer with a K_D equal to 1.6 nM at 11 °C. Isoelectric focusing patterns reveal the presence of at least 12 different forms of dimeric SBP molecules probably resulting from the presence of different amounts or types of carbohydrate side chains. The data indicate a very close similarity in molecular and steroid-binding properties to human SBP and establish the macaque monkey as a valuable animal model to study the physiological role of SBP in humans.

A sex steroid binding protein (SBP)[1] has been detected in the plasma of many species, including humans (Mercier et al., 1966; Rosenbaum et al., 1966; Pearlman & Crepy, 1966), many nonhuman primates (DeMoor et al., 1969; Petra & Schiller, 1977; Renoir et al., 1980), cows, (Murphy, 1968; Corvol & Bardin, 1973), rabbits, goats, and sheep (Corvol & Bardin, 1973), reptiles and amphibians (Murphy, 1968; Ozon et al., 1971; Corvol & Bardin, 1973), fishes (Idler & Freeman, 1968; Corvol <<am Bardin, 1973), dogs (Carstensen et al., 1974; Tabei et al., 1978), and cats (Mickelson, 1977). The data indicate that all primate SBPs bind 5α-dihydrotestosterone, testosterone, and 17β-estradiol with high affinity whereas SBPs from other species are mainly androgen-binding proteins. Furthermore, antibodies raised against human SBP (hSBP)[2] cross-react with all primate SBPs but not with any SBP isolated from other species (Bordin et al., 1978; Petra, 1979; Renoir et al., 1980).

Human SBP was the first to be purified to homogeneity (Mickelson & Petra, 1975) followed by bovine SBP (Suzuki et al., 1977), rabbit SBP (Mickelson & Petra, 1978), and canine SBP (Suzuki et al., 1979). The experimental results obtained from chemical characterization of these various forms of SBP indicate that the native protein has a molecular weight of 80 000–90 000[3] and is composed of two similar, if not identical, subunits (Michelson et al., 1978; Mickelson & Petra, 1978; Petra, 1979; Petra et al., 1983). In the case of rabbit SBP, this conclusion has also been recently confirmed by others (Kotite & Musto, 1982; Danzo et al., 1982).

The investigation of the physiological role of SBP in human plasma necessitates the development of an adequate animal model. We have recently reported preliminary results indicating an inverse relationship between SBP and the metabolic clearance rate of testosterone in the rhesus monkey (Petra et al., 1983). These data were obtained by intravenous infusion of homogeneous hSBP, rhSBP, and purified monospecific hSBP antibodies. The macaque had previously been proposed as an ideal model for understanding the physiological role of SBP in humans because the steroid-binding properties of both human and macaque SBPs were almost identical (Petra & Schiller, 1977; Petra, 1979; Renoir et al., 1980). However, more data were needed on the molecular characteristics of macaque SBP for comparison with human SBP before the monkey model could be firmly established. This paper describes the purification procedure and characterization of two SBPs isolated from *Macaca mulatta* and *Macaca nemestrina* sera and compares the physicochemical properties to those of the human protein. Because the physiological studies mentioned above required large amounts of homogeneous *Macaca mulatta* SBP, a more complete characterization could only be realized for *Macaca nemestrina* SBP. As expected, however, the two macaque SBPs were found to be similar in all aspects studied.

Materials and Methods

Chemicals. Macaca nemestrina serum was obtained from males and females at the Regional Primate Center at the University of Washington. *Macaca mulatta* (rhesus) serum was drawn from females at the Oregon Regional Primate Center. Human pregnancy serum collected at term was obtained from the delivery room in the department of obstetrics and Gynecology at the University of Washington School of Medicine. All chemicals used have been described in previous publications (Mickelson et al., 1978; Mickelson & Petra, 1978; Petra & Lewis, 1980).

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[1] This nomenclature was first adopted at the IVth Meeting of the International Study Group for Steroid Hormones, Rome, 1969. Subsequently, other terms have been introduced in the literature. These are the following: steroid-binding β-globulin, SβBG; sex hormone binding globulin, SHBG; testosterone-binding globulin, TBG; estradiol-binding protein, EBP; testosterone–estradiol-binding globulin, TeBG. The latter three terms are somewhat misleading because the protein actually binds 5α-dihydrotestosterone better than testosterone or 17β-estradiol, and in rabbits and other nonprimate species, 17β-estradiol is not bound significantly under physiological conditions.

[2] Abbreviations: SBP, sex steroid binding protein; hSBP, human SBP; rSBP, rabbit SBP; nSBP, *Macaca nemestrina* SBP; rhSBP, rhesus SBP (*Macaca mulatta*); DHT, 5α-dihydrotestosterone; DMF, dimethylformamide; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; PAGE, polyacrylamide gel electrophoresis.

[3] The molecular weight of 120K for native hSBP we reported earlier (Mickelson et al., 1978) was obtained by gel filtration. Abnormally high molecular weights have been found for glycoproteins by using this method as stated in that paper. Values obtained for the native protein by equilibrium sedimentation are to supersede all other values previously reported by our laboratory.

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Assay of SBP. The binding activity of SBP was measured by the DEAE-cellulose filter assay with [^{3}H]DHT as ligand (Mickelson & Petra, 1974). A 70% filter assay "efficiency" was used throughout this paper. Radioactivity was counted in a Beckman LS-100C scintillation counter with 4 mL of Omnifluor (New England Nuclear).

Siiteri et al. (1982) have recently claimed that it is unnecessary to correct for loss of specific binding during the performance of the filter assay as previously found (Mickelson & Petra, 1974). These investigators assume that 100% of the steroid–protein complexes are retained by the DEAE filter during application and washing procedures. This assumption is incorrect. Binding of complexes to the DEAE filters is controlled by an equilibrium which depends upon the charge capacity and hydration of the filter. We therefore urge those laboratories using this assay to continue estimating their own filter efficiencies and to correct for losses. Failure to do so will result in underestimated values.

Analytical Methods. Amino acid analyses were determined in a Durrum Model D-5090 amino acid analyzer after hydrolysis in 0.2 mL of 5.7 N HCl at 110 °C for 24, 48, and 96 h in evacuated tubes. norleucine was included for quantitation of losses. Isoleucine and valine values were calculated from the 96-h hydrolysis, and serine and threonine values were extrapolated to zero time of hydrolysis. Cystine and cysteine were determined as cysteic acid after performic acid oxidation as described by Moore (1963). Tryptophan content and extinction coefficient were determined spectrophotometrically in the presence and absence of 6 M guanidine hydrochloride (Edelhoch, 1967). The molar concentration of SBP was determined in two ways: first, by amino acid analysis with norleucine standards using 62 nmol of aspartic acid per dimeric nSBP and 60 nmol of aspartic acid per dimeric hSBP; second, by weighing the lyophilized powder after drying in vacuo at 100 °C for 24 h. The nSBP and hSBP molecular weights used for these calculations were 88 000 (77 440 for the dimeric polypeptide) and 88 000 (75 680 for the dimeric polypeptide), respectively. Absorbance spectra were measured at 25 °C in 1-cm quartz cuvettes in a Cary 15 spectrophotometer. Uncorrected fluorescence spectra were obtained at 25 °C in a Perkin-Elmer MPF-44A spectrofluorometer equipped with a thermostated cuvette holder and a 1-cm quartz cuvette. Both excitation and emission bandwidths were set at 4 nm. Excitation was at 295 nm to avoid contribution from tyrosine. Carbohydrate composition was determined by gas chromatography and mass spectroscopy after hydrolysis in 90% acetic acid containing 0.5 N H_2SO_4 as described by Carter & Hakamori (1979). Sialic acid was determined by the thiobarbituric acid method of Warren (1959).

Purification of SBP. Purification of macaque SBP was carried out as described earlier for human SBP (Petra & Lewis, 1980) with modifications. Frozen serum (1–1.5 L) was thawed at 4 °C, adjusted to 0.1 M sodium phosphate, pH 6.8, and centrifuged for 1 h at 16000*g*. The pellet and a lipidlike material which collected at the surface were discarded. Ammonium sulfate was added gradually to a concentration of 50%. Protein precipitate was removed by centrifugation at 16000*g* for 20 min and the supernatant discarded. The pellet, containing about half the original protein, was dissolved in half the original serum volume of 0.01 M Tris-HCl, 10% (v/v) glycerol, and 10% (v/v) DMF, pH 7.4. This solution was centrifuged at 40000*g* for 30 min, the pellet discarded, and the supernatant (about 400 mL) incubated at 4 °C overnight directly with 100 mL of packed 5α-dihydrotestosterone 17α-hexanyldiaminoethyl(1,4-butanediol diglycidyl ether)–agarose to adsorb the SBP. The gel suspension was thoroughly washed 6 times on a sintered-glass funnel by resuspension in 5 volumes of 10 mM Tris-HCl, 10% glycerol, and 10% DMF, pH 7.4, plus 0.5 M NaCl in every second wash. The gel was then incubated overnight at 4 °C with 500 mL of 10 mM Tris–HCl, 10% (v/v) glycerol, 0.1 M NaCl, 5 mM $CaCl_2$, and 2×10^{-5} M DHT, pH 7.4. The gel was filtered and the filtrate concentrated by ultrafiltration on a PM-10 membrane (Amicon Corp.) to less than 4 mL. The final step for obtaining pure SBP was preparative gel electrophoresis as previously described (Mickelson et al., 1978). Human SBP was purified as previously reported (Petra & Lewis, 1980) with the inclusion of the ammonium sulfate precipitation step described above prior to DEAE-cellulose chromatography. Ion-exchange chromatography was found unnecessary in the macaque SBP purification procedure and was omitted in order to increase the final yield of pure protein. Pure SBP was stored frozen at −20 °C in 10 mM Tris-HCl, 0.1 M NaCl, 5 mM $CaCl_2$, 10% (v/v) glycerol, and 2×10^{-5} M DHT, pH 7.4.

Molecular Weight Determination. The molecular weight of native macaque SBP was determined by electrophoresis in 4% *T*–20% *T*(1% *C*) gradient polyacrylamide slab gels (178 × 2.7 × 110 mm) in 10% glycerol and 0.12 M Tris-HCl, pH 8.9, where *T* refers to the total percent monomer concentration [acrylamide + bis(acrylamide)] and *C* refers to the concentrations of bis(acrylamide) expressed as a percentage of *T*. The electrophoresis buffer was 0.05 M Tris–0.19 M glycine, pH 8.3. Electrophoresis was carried out at 4 °C and 80 V for 1–12 h according to Lambin & Fine (1979) with the following protein standards: 669K, thyroglobulin; 440K, ferritin; 232K, catalase; 140K, lactic acid dehydrogenase; 88K, human SBP; 80K, rabbit SBP; 70K, human transferrin.

Subunit molecular weights were obtained in 10% *T*–3% *C* slab gels with a 5% *T*–3% *C* stacking gel in the presence of 0.1% SDS in Tris buffers as previously described (Weber & Osborn, 1975). Molecular weights of native and SDS-treated SBPs were obtained by plotting log or *M* vs. R_f, using the following protein standards: 94K, rabbit phosphorylase *b*; 67K, bovine serum albumin; 43K, bovine carbonic anhydrase; 30K, soybean trypsin inhibitor; 14.4K, bovine α-lactalbumin.

Determination of Equilibrium Binding Constants. The equilibrium constants of DHT dissociation from pure nSBP and hSBP were determined by the steady-state gel electrophoresis procedure (Petra & Schiller, 1977; Tabei et al., 1978) as originally described by Ritzen et al. (1974). The equilibrium constant, K_D, was determined indirectly by using the following equation derived from the law of mass action:

$$BP_{tot} = S_b[(K_D/[S_u]) + 1]$$

where K_D is the dissociation constant, $[S_u]$ the concentration of unbound steroid, S_b the mole amount of steroid bound, and BP_{tot} the maximum mole amount of steroid-binding sites. BP_{tot} is obtained experimentally by a saturation analysis performed in polyacrylamide gels at increasing concentrations of [^{3}H]-DHT (0.2–4.5 nM). The equilibrium constant is calculated by substituting BP_{tot}, $[S_u]$, which is the concentration of [^{3}H]DHT per gel that is essentially equal to the unbound steroid, and S_b, which is the amount of radioactivity in the SBP peak minus base-line counts in the gel. Glass tubes (140 mm × 5 mm i.d.) were filled up to 120-mm height with a 5% *T*–3% *C* polyacrylamide separating gel prepared in 0.38 M Tris-HCl–10% glycerol, pH 8.9, containing the proper amount of [^{3}H]DHT. All pHs were measured at room temperature. All gels were "topped" with a stacking gel(4% *T*–2% *C*) prepared in 0.05 M Tris-HCl–10% glycerol (v/v), pH 6.7, containing about 2 nM [^{3}H]DHT. The gel slices were allowed

Table I: Purification Scheme of *Macaca mulatta* SBP[a]

step	total protein (mg)[b]	sp act.[c]	% yield at each step	% total yield
serum (1350 mL)	95 000	0.35	100	100
$(NH_4)_2SO_4$ precipitation	45 000	0.64	88	88
affinity chromatography	36			
preparative PAGE	4.2	3314	90	42

[a] Same method and similar yields for *Macaca nemestrina* SBP. [b] Measured according to Lowry et al. (1951) except for pure SBP which was measured by using ϵ_{280} in Table III. [c] Expressed as nanograms of DHT bound per milligram of protein measured by the filter assay (Mickelson & Petra, 1974). The presence of radioinert DHT at the affinity chromatography step precluded assay of SBP by the filter assay.

to extract at least 24 h in the Omnifluor scintillant before counting. Underestimated values of S_b will be obtained if this step is omitted. Pure SBP was diluted with 0.05 M Tris-HCl, 10% (v/v) glycerol, and 2×10^{-5} M DHT, pH 6.7, and 50 μL (0.2–0.3 pmol of SBP) was applied directly to gels. The top buffer chamber was maintained at 20 °C in the early stages of electrophoresis to promote rapid steroid exchange, which in the case of primate SBPs is very slow at lower temperatures. The bottom buffer chamber, which surrounded the lower two-thirds of the gels where SBP steroid exchange reaches a steady state, was maintained at 11 °C. Electrophoresis was for 6 h starting at 2 mA/gel, with a constant power of 3 W and a voltage limit of 200 V. Determination of K_D by this procedure had been shown feasible in the case of pure rSBP (our unpublished results; Kotite & Musto, 1982).

Isoelectric Focusing. Isoelectric focusing was performed in a Bio-Rad Model 1415 slab electrophoresis cell as described by the manufacturer with a Pharmacia Model ECPC 2000 power supply. Gels consisted of a pH 3.5–10 gradient in 5% *T*–3% *C* acrylamide, 5% glycerol, 2% ampholine (LKB), 0.005 mg/mL riboflavin 5′-phosphate, and 0.1 mg/mL ammonium persulfate. Wells were formed to accommodate 50-μL samples (50 μg of SBP). Standards used were human carbonic anhydrase B (p*I* 6.5), β-lactoglobulin (p*I* 5.13, 5.23), and soybean trypsin inhibitor (p*I* 4.5). Focusing was for 1.5 h at 30 W and 2 °C. Protein bands no longer migrated under these conditions.

Immunochemical Methods. Preparation and purification of antibodies and immunoelectrophoresis were carried out as previously described (Bordin et al., 1978; Bordin & Petra, 1980).

Results

Purification of SBP. Table I summarizes the purification scheme of *Macaca mulatta* SBP. About a 10 000-fold purification is needed to obtain a 40% yield of pure protein. The procedure and results were similar for *Macaca nemestrina* SBP. As in the case of hSBP purification (Petra & Lewis, 1980), affinity chromatography is critical for obtaining large amounts of pure protein efficiently.

Characterization of SBP. Because of the low amount of protein available, it was not possible to estimate the native molecular weight of *Macaca nemistrina* SBP by equilibrium sedimentation. Instead, the electrophoretic method of Lambin & Fine (1979) was used under native conditions in gradient polyacrylamide gels. This method is subject to large errors (mean deviation of ±25% for a total of 10 standards) when unrelated proteins are used as standards (Lambin & Fine, 1979). For instance, transferrin and thyroglobulin were found

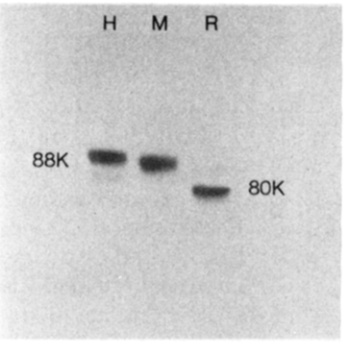

FIGURE 1: Determination of the native molecular weight of *Macaca nemestrina* SBP by electrophoresis in 4–20% gradient polyacrylamide gels. The figure represents the 12-h end point where electrophoretic migration remains constant. The gel was prepared in 10% glycerol and 0.12 M Tris-HCl, pH 6.7. Electrophoresis was carried out at 80 V and 4 °C in 0.05 M Tris-HCl–0.19 M glycine, pH 8.3. Protein samples of 10 μg were applied. Molecular weights of human and rabbit SBP standards were estimated by equilibrium sedimentation (Petra et al., 1983). H, human SBP; M, *Macaca nemestrina* SBP; R, rabbit SBP.

to have +14% and −31% deviations from literature values, respectively. These observations were confirmed in this study where transferrin, hSBP, and rSBP were found to yield molecular weights over 100K when thyroglobulin (669K), ferritin (440K), catalase (232K), lactic acid dehydrogenase (140K), and bovine serum albumin (67K) were used as standards in the Lambin–Fine procedure. This anomaly was also recently reported by Kotite & Musto (1982), who found a molecular weight value of 101 600 for rSBP as compared to 80 000 by sedimentation equilibrium (Petra et al., 1983). This difference corresponds to an error of +25%, which is within the predicted mean error deviation of the Lambin–Fine procedure. In order to minimize this error, the species variants hSBP and rSBP were used as standards to estimate the molecular weight of nSBP. Monkey SBP migrated with R_fs similar to those of hSBP in every case when electrophoresis was performed for different lengths of time. Figure 1 represents the 12-h end point, at which time all three SBPs stop migrating. We therefore assign a value of 88K for the native molecular weight of nSBP. There will be an error associated with this value, but it is estimated to be much less than 10% because we could clearly differentiate between the two bands of hSBP and rSBP in Figure 1, the molecular weights of which appear to vary by 9%.

The subunit molecular weight of the major band of nSBP was estimated to be 47 000 by SDS gel electrophoresis as shown in Figure 2. The corresponding value for the major band of hSBP is 44 000.

The isoelectric focusing pattern of nSBP, shown in Figure 3, is different from that of hSBP but also shows extensive microheterogeneity most likely caused by differences in covalently bound carbohydrate side chains. *Macaca nemestrina* SBP is composed of about 12 bands focusing from p*I* 5.1 to 5.6 with principal bands at p*I* 5.2 and 5.3. The bands of hSBP are slightly more basic and focus from p*I* 5.3 to 5.9.

The amino acid and carbohydrate compositions are shown in Table II. The hSBP composition previously reported (Mickelson et al., 1978; Mickelson & Petra, 1978; Petra, 1979) has been corrected and recalculated to include newly acquired data from 15 different analyses, and both compositions are now expressed on the basis of the dimeric polypeptide molecular weight. The amino acid analyses indicate that there are 6–8 mol of tyrosine per mol of dimer. Thus, with an extinction coefficient of 1280 cm^{-1} M^{-1} (Edelhoch, 1967), the

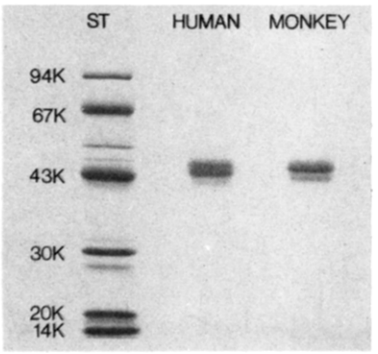

FIGURE 2: Determination of the subunit molecular weight of SBP by SDS–polyacrylamide gel electrophoresis in discontinuous buffers at 11 °C. The stacking gel (5%) was in 0.1% SDS–0.5 M Tris-HCl, pH 6.8, and the separating gel (10%) in 0.1% SDS–1.5 M Tris-HCl, pH 8.8. The electrode buffer consisted of 0.025 M Tris, 0.192 M glycine, and 0.1% SDS, pH 8.3. Ten micrograms of SBP (10 μL) was added to 15 μL of H_2O and 25 μL of SDS buffer (0.125 M Tris-HCl, 4% SDS, 10% β-mercaptoethanol, and 48% urea, pH 6.8) and heated at 100 °C for 5 min. The voltage was set at 60 V until dye migrated into the separating gel and then was increased to 200 V for 4 h. The standard proteins are listed under Materials and Methods.

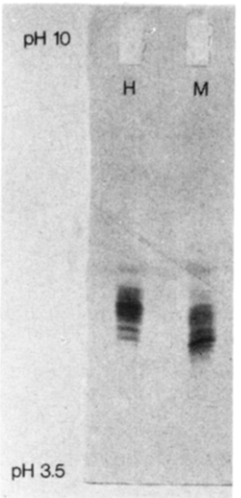

FIGURE 3: Analytical isoelectric focusing of SBP in thin layers of polyacrylamide gels. Fifty micrograms (50 μL) was dialyzed against H_2O and placed in the wells shown in the gel. Constant power was at 30 W and 2 °C for 1.5 h at which time SBP bands and protein standards (now shown) were focused. H, human SBP; M, *Macaca nemestrina* SBP.

contribution of tyrosine to the absorption maximum at 282 nm is less than 8%. At 288 nm, the maximum tyrosine contribution is less than 3%, which is smaller than the experimental uncertainty of the spectral measurements. Hence, ignoring the tyrosine contribution, we calculate a value of 22 tryptophan residues per mol of dimer SBP for each of the primate proteins at 288 nm in 6 M guanidine hydrochloride. This represents a correction in tryptophan content which we had reported earlier for hSBP (Mickelson et al., 1978). The carbohydrate content of hSBP was also found to be 14% instead of 18%. A parallel but less complete analysis of rhSBP showed no significant differences when compared to nSBP.

Spectral Characteristics. The spectral properties of nSBP and hSBP are shown in Figure 4. The molar extinction coefficients at 280 nm, ϵ_{280}, were calculated to be 1.10×10^5 cm^{-1} M^{-1} for nSBP and 1.14×10^5 cm^{-1} M^{-1} for hSBP. The human value was derived by averaging the result obtained by amino acid analysis (1.33×10^5 cm^{-1} M^{-1}) with that obtained by weighing the protein (0.96×10^5 cm^{-1} M^{-1}) as described under Materials and Methods. The latter procedure involves

Table II: Amino Acid and Carbohydrate Compositions[a]

	nSBP[b]	hSBP[c]
cysteic acid	7.3	7.6
aspartic acid	62.8	60.8
threonine	26.5	35.9
serine	63.1	64.0
glutamic acid	83.0	70.0
proline	50.5	55.6
glycine	70.0	73.0
alanine	51.2	46.1
valine	44.4	35.9
methionine	11.6	7.7
isoleucine	12.0	19.3
leucine	94.2	90.2
tyrosine	8.0	6.0
phenylalanine	24.0	20.6
histidine	18.3	20.9
lysine	26.2	24.5
arginine	33.7	30.2
tryptophan	22.0	22.0
mannose	10.8	11.3
galactose	10.8	13.6
N-acetylglucosamine	18.0	19.4
N-acetylgalactosamine	2.2	3.0
fucose	1.2	1.1
sialic acid	9.8	9.6

[a] Compositions given as moles per mole of SBP dimer. [b] Calculated on the basis of a dimeric polypeptide of M_r 77 440. [c] Calculated on the basis of a dimeric polypeptide of M_r 75 680.

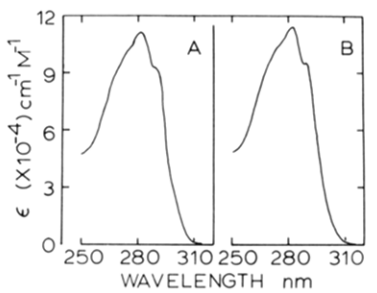

FIGURE 4: Ultraviolet absorption spectra of human and monkey SBP. Protein was dialyzed against 10 mM Tris-HCl, 10% glycerol, 0.1 M NaCl, 5 mM $CaCl_2$, and 2×10^{-5} M DHT, pH 7.4. (A) *Macaca nemestrina* SBP; (B) human SBP.

drying the lyophilized powder at 100 °C in vacuo for 24 h to remove most of the bound water. This treatment resulted in a 10% loss of weight, which corresponds well with values of bound water obtained for other proteins. The calculated value of 0.95×10^5 cm^{-1} M^{-1}, however, will reflect a minimum estimation of ϵ_{280} because SBP is a glycoprotein and the small amount of water remaining bound to the sugar portion of the molecule may be more difficult to remove than that bound to the polypeptide chain under the conditions used. The average between the two values will therefore represent a more accurate estimation of ϵ_{280}. The same correction was applied to nSBP because of the similarity in spectral properties, sugar content, and molecular weight between the two proteins.

The fluorescence emission spectrum of nSBP excited at 295 nm, where the absorption of tyrosine is negligible, has a maximum at 336 nm. The fluorescence emission spectrum of nSBP is similar to that of hSBP. For comparison, the emission spectrum of *N*-acetyltryptophanamide, which serves as a model for fully solvated or "exposed" tryptophan residues, is red shifted 14 nm to a maximum of 350 nm. This implies that the emissive tryptophan residues of both proteins are in a hydrophobic or solvent-restricted environment.

Steroid-Binding Properties of nSBP and hSBP. Scatchard analyses of pure nSBP and hSBP are shown in Figure 5. The equilibrium constants of DHT dissociation are not obtained

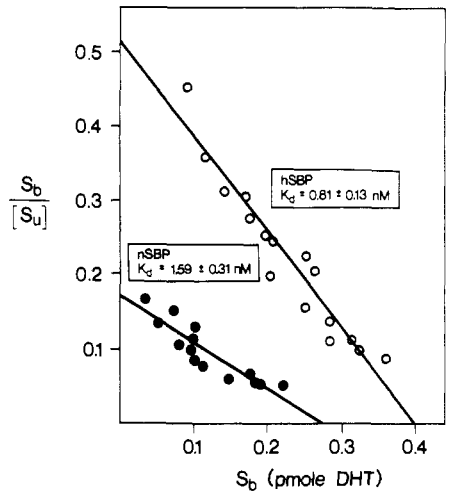

FIGURE 5: Determination of the equilibrium binding constant of DHT dissociation by steady-state gel electrophoresis as described under Materials and Methods. The apparent equilibrium constant is an average of the equilibrium constants calculated at each point by using the equation described under Materials and Methods. The total amount of nSBP and hSBP applied to the gels was 0.31 pmol in both cases as determined spectrally.

directly from the slopes of these plots as done conventionally but are calculated from the equation described under Material and Methods where BP_{tot} and S_b are expressed in total mole amounts instead of concentrations. This is necessary because it is not possible to determine the molar concentration of bound SBP in the polyacrylamide gels. The K_Ds for nSBP and hSBP were calculated to be 1.59 ± 0.31 and 0.81 ± 0.13 nM at 11 °C, respectively. These values agree fairly well with those determined at 4 °C in *Macaca nemestrina* serum, K_D = 0.53 nM (Petra & Schiller, 1977), and in human serum, K_D = 0.42 nM (Petra, 1979), also determined at 4 °C. Both proteins are almost identical in steroid-binding specificity as previously determined in serum (Petra & Schiller, 1977).

The stoichiometry of steroid binding is determined by comparing the extrapolated values at the abscissa of both lines in Figure 5, which represent the total amount of steroid-binding sites present in the electrophoresed SBP bands, to the total amount of SBP applied to the gels as determined spectrally. The nSBP results yield 0.27 pmol of steroid-binding sites per 0.31 pmol of nSBP or 0.87 mol of DHT bound per mol of dimeric nSBP. The hSBP results yield 0.40 pmol of steroid-binding sites per 0.31 pmol of hSBP or 1.32 mol of DHT bound per mol of dimeric hSBP. The results therefore indicate that, within experimental error, dimeric SBP binds one molecule of steroid, assuming that the pure protein did not lose DHT-binding activity after the affinity chromatography step.

Immunochemical Properties of nSBP. *Macaca nemestrina* serum was previously shown to cross-react with rabbit anti-human SBP but not with guinea pig anti-rabbit SBP (Bordin & Petra, 1978). The same results were obtained with pure nSBP.

Discussion

The results presented here indicate that the molecular and steroid-binding properties of macaque SBP are very similar to those of human SBP as shown in Tables II and III. This includes the molecular weights of the native and denatured proteins, the amino acid and carbohydrate compositions, the binding affinity of DHT and the stoichiometry of steroid binding, the electrophoretic mobility, and the spectral characteristics. Because of these similarities, nSBP can be purified

Table III: Physicochemical Properties of SBP

	nSBP	hSBP
native mol wt	88 000	88 000
monomeric mol wt	47 000	44 000
K_D (DHT) at 11 °C (nM)	1.59	0.81
ϵ_{280} ($\times 10^5$ cm^{-1} M^{-1})	1.10	1.14
steroid-binding sites per dimer[a]	1	1
carbohydrate content (%)	12	14

[a] Assuming no loss of DHT-binding activity subsequent to the affinity chromatography step.

by the same method used for hSBP, thereby eliminating the need for devising new procedures to obtain significant quantities of pure protein. There are, however, subtle differences between the two proteins which appear to arise from differences in the distribution of carbohydrate side chains. This is shown in Figure 3, which demonstrates that the isoelectric focusing pattern of nSBP is different from that of hSBP. In the latter case, we have already demonstrated that each band represents an active dimeric form of hSBP since all the bands are radioactive when the sample is incubated with [^{3}H]DHT prior to focusing (Petra et al., 1983). As shown in this paper, 1 mol of steroid is bound per dimer; therefore, all the focused bands must represent the dimeric form of the protein. Neuraminidase treatment prior to isoelectric focusing reduces the number of bands, and *all* those which remain focus at a less acidic pH (unpublished results), suggesting that all the species contain carbohydrate side chains terminating with sialic acid residues. Most of the microheterogeneity observed in the focusing patterns of nSBP and hSBP in Figure 3 must therefore be attributed to differences in the content or type of carbohydrate side chains from one molecule of SBP to the next. It should also be mentioned that slight differences in amide content as well as amino acid sequence between the two proteins produced by allotypic replacements may also account for differences in the focusing patterns.

The microheterogeneity visualized in the SDS gel electrophoretic pattern of Figure 2 can also be explained as being attributed to differences in carbohydrate content. One interpretation for the existence of the major and minor bands[4] is to propose that native SBP is composed of three subunits, one represented by the minor band and the other two migrating together in the major band. This proposal, however, would require that native SBP have a molecular weight of at least 120K in order to account for all three subunits. We have recently ruled out this possibility by equilibrium sedimentation studies in the analytical ultracentrifuge which show that native hSBP has a molecular weight of 88 000 (Petra et al., 1983). We therefore conclude that the microheterogeneity shown in Figures 2 and 3 arises through the combination of many different forms of the same two subunits, forms which for the most part are generated by the attachment of different amounts or types of carbohydrate side chains. This combination gives rise to a large number of different dimeric forms of active SBP molecules which can be visualized by isoelectric focusing. The SDS pattern of Figure 2 is not as extensive as the isoelectric pattern of Figure 3 because differences in molecular weight between the various dimeric species of SBP are for the most part not significant enough to be detected by SDS gel electrophoresis. Earlier studies in human serum had suggested that the microheterogeneity of SBP was due to carbohydrate variability (Van Baelen et al., 1969). These

[4] In the case of hSBP, the content and migration of the minor band have been found to vary from preparation to preparation.

authors also showed that partial removal of sugar side chains did not influence the steroid-binding activity of serum The data presented here not only confirm that conclusions made at the serum level also apply to the pure protein but also indicate that the subtle differences in sugar content between hSBP and nSBP do not affect steroid-binding activity since both proteins have similar K_Ds. The data also demonstrate that nSBP and hSBP have the same molecular arrangement, composed of two similar subunits bound in a specific configuration to recognize one molecule of steroid, as previously suggested for hSBP (Petra et al., 1983).

In summary, the molecular characteristics of *Macaca mulatta* SBP and *Macaca nemestrina* SBP have been sufficiently investigated to be compared to those of human SBP. The results show a very close similarity in molecular and steroid-binding properties between the three proteins. The results establish the macaque monkey as a valuable animal model to study the physiological role of SBP in humans.

Added in Proof

While this paper was in press, Cheng et al. (1983) reported a molecular weight of 115K for native hSBP. This value is much higher than our published value of 88K. These investigators, however, used the Lambin–Fine electrophoretic method, which yields overestimated values by at least 25% for both hSBP and rSBP as shown here. These authors also quote the 94K value found by Rosner & Smith (1975) to compare with and support their 115K value. The 94K value was determined by SDS–PAGE and therefore cannot represent hSBP which migrates at 44K in SDS–PAGE. Instead, the 94K material corresponds to a major protein impurity. The most refined values recently calculated from six different equilibrium sedimentation experiments are 85 440 ± 3360 (SD) for hSBP and 85 750 ± 5290 (SD) for rSBP (unpublished results).

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