Photoaffinity Labeling of Homologous Met-133 and Met-139 Amino Acids of Rabbit and Sheep Sex Hormone-Binding Globulins with the Unsubstituted Δ^6-Testosterone Photoreagent†

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ABSTRACT: Purified rabbit and sheep sex hormone-binding globulins (SHBGs) were photolabeled by Δ^6-testosterone. The maximal levels of specific incorporation were respectively 0.33 and 0.30 mol of label/mol of homodimer. Tryptic cleavage of photolabeled SHBGs gave a single radioactive peptide for rabbit SHBG and two major radioactive peptides S_1 and S_2 for sheep SHBG. Edman sequencing of the photolabeled peptide of rabbit SHBG revealed a single sequence corresponding to peptidic fragment Leu-118-Lys-134. Subcleavage of this peptide with elastase led to a single radioactive peptidic fragment corresponding to dipeptide Met-133-Lys-134, identified by mass spectrometry, while deletion of the C-terminal residue with carboxypeptidase B showed that all the radioactivity remained on peptide Leu-118-Met-133, thus demonstrating that photolabeling occurred exclusively on Met-133, the only residue common to the two radioactive subcleaved peptides. Edman sequencing of peptides S_1 and S_2 of sheep SHBG showed a same single sequence corresponding to residues Gln-126-Arg-140 which contained no identifiable phenylthiohydantoin derivative at cycle 14, thus indicating that in both cases the corresponding Met-139 residue is the main site of photolabeling, as confirmed for peptide S_1 by the presence at this cycle of a major peak of radioactivity while in peptide S_2 the photoattachment of Δ^6-testosterone was found labile in the conditions of sequencing. The photolabeled peptide S_1 was characterized by mass spectrometry which showed the covalent fixation of one mole of Δ^6-testosterone and the presence of a biantennary oligosaccharide attached at Asn-133, which suggests that the steroid-binding site is probably not deeply buried in the SHBG homodimer.

The sex hormone-binding globulin (SHBG),[1] also called sex steroid-binding protein (SBP), is a transport glycoprotein present in the plasma of most mammalian species which binds 5α-dihydrotestosterone (DHT), testosterone (T), and, in some species, estradiol (E_2). The major biological role of SHBG is the regulation of the free steroid concentration of the active sex steroids while more recent studies have suggested the presence in several target cells of membrane SHBG receptors involved in internalization processes or in steroid-mediated effects on cAMP levels (cf. refs *1*−*3*). SHBG is a homodimeric glycoprotein with only one steroid-binding site per mole of homodimer. The monomer of human SHBG is a single polypeptide of 373 residues (*4*−*7*) whereas the rabbit SHBG monomer is shorter by six or seven residues at the amino terminal (*8*, *9*). Human and rabbit SHBGs share 79.0% of amino acid identity. Recently, the cDNA nucleotide sequence of sheep SHBG revealed that this protein comprises 373 residues which share 80% and 73% of amino acid identity with the corresponding human and rabbit proteins (*10*).

Human SHBG contains one O-linked and two N-linked oligosaccharides (*11*) at Thr-7 and at Asn-351 and Asn-367, respectively (*4*), whereas rabbit SHBG is only N-glycosylated at the two conserved homologous Asn-345 and Asn-361 consensus sites. Sheep SHBG contains three consensus sites of N-glycosylation, one at Asn-367 conserved in all species studied, and two others at Asn-33 and Asn-133 which are not conserved loci (*10*). The absence of significant differences in the binding characteristics of enzymatically deglycosylated human or rabbit SHBGs (*12*) and unglycosylated mutants of human SHBG (*13*), as compared with those of the corresponding native SHBGs, suggests that the oligosaccharide chains do not interfere with steroid binding.

Although human and rabbit SHBGs have similar primary sequences, the half-dissociation time at 0 °C of the plasmatic human SHBG−DHT complex ($t_{1/2}$ = 70 min) (*14*) is longer than that of the rabbit SHBG−DHT complex ($t_{1/2}$ = 5.2 min)

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[1] Abbreviations: ABP, androgen-binding protein; BSA, bovine serum albumin; Δ^6-T, 17β-hydroxyandrosta-4,6-dien-3-one; Δ^6-E_2, estra-1,3,5-(10),6-tetraene-3,17β-diol; DCC, dextran-coated charcoal; DHT, 5α-dihydrotestosterone; DEAE, (diethylamino)ethyl; E_2, estradiol; HPLC, high-performance liquid chromatography; $NaDodSO_4$, sodium dodecyl sulfate; PTH, phenylthiohydantoin; PBS, phosphate-buffered saline; SBP, sex binding protein; SHBG, sex hormone-binding globulin; T, testosterone; TFA, trifluoroacetic acid.

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(*15*), a result consistent with the higher binding affinity of DHT for human SHBG than for rabbit SHBG (*9, 16*). On the other hand, human SHBG also binds E_2 although with a weaker affinity than for androgens, whereas rabbit and ram SHBGs were reported to be almost totally specific for androgens (*15, 17*−*19*).

Affinity labeling experiments have suggested that a large part of the steroid-binding site of human SHBG is located in the region Lys-134-Met-139 which contains a conserved Met-139 residue photolabeled by androgenic Δ^6-testosterone (Δ^6-T) or estrogenic Δ^6-estradiol (Δ^6-E_2) photoreagents (*20, 21*) and a Lys-134 residue selectively alkylated with 17β-bromoacetoxy-[^{14}C]DHT (*22*), whereas rat androgen-binding protein (ABP) was photolabeled by Δ^6-T on the peptidic fragment Ile-141-Ser-151 (*23*). Site-directed mutagenesis experiments (*24*−*27*) have confirmed the importance of the Met-139 residue for steroid binding by human SHBG and have demonstrated that the steroid-binding site is mainly situated within the Leu-1-Glu-205 portion of human SHBG which comprises the above-described sites of affinity labeling.

Although the Met-139 residue of human SHBG is conserved in SHBG or ABP of all species studied, the region immediately N-terminal of Met-139 is poorly conserved and may contain residues important for explaining the differences in steroid-binding specificity among the species, as shown by individual or complete substitutions of amino acids between human SHBG and rat ABP which modified binding affinities for estradiol without changing significantly those for DHT (*28, 29*). However, none of the mutants could fully acquire the profile of binding properties of the protein corresponding to the inserted residues, thus suggesting that the peptide 130−138 may not be the only factor controlling the specificity of the steroid-binding site. By contrast, the hydrophobic region immediately C-terminal of Met-139 is almost entirely conserved between species. Amino acid substitutions within this region led to mutants with unchanged binding affinity for DHT but decreased ability for dimerization (*28*).

Therefore, rabbit and sheep SHBGs were chosen as models to determine whether the corresponding conserved methionine residues homologous to Met-139 of human SHBG still remain in the vicinity of the steroid ligand despite the differences in amino acid sequences of the peptide segment corresponding to the nonconserved region Pro-130-Ile-138 of human SHBG and the presence, for sheep SHBG, of a consensus site of N-glycosylation at Asn-133 in the vicinity of the presumed steroid-binding site, which, if glycosylated, may influence the steroid-binding properties. In the present work we report the covalent photolabeling of purified rabbit and sheep SHBGs with the Δ^6-[^{3}H]T photoreagent previously employed for the photolabeling of human SHBG (*21*).

EXPERIMENTAL PROCEDURES

Chemicals. [1,2,4,5,6,7-$^3H_6$]DHT (126 Ci/mmol) was purchased from Amersham Corp., and [1α,2α-$^3H_2$]T (53.5 Ci/mmol) was from NEN. DHT, T, and E_2 were from Roussel-UCLAF (Paris, France), and 2-methoxyestrone was from Sigma and was reduced to 2-methoxyestradiol using $NaBH_4$ in ethanol. L-1-(Tosylamido)-2-phenylethyl chloromethyl ketone trypsin (TPCK-trypsin) was from Worthington. Porcine pancreatic elastase, carboxypeptidase B, and recombinant *N*-glycosidase F were from Boehringer (Manheim, Germany). Trisacryl GF 05 was from Biosepra (Villeneuve-la-Garenne, France). The protein assay reagent (BCA protein assay) was purchased from Pierce.

Buffers. PBS (10 mM sodium phosphate buffer, pH 7.4, and 0.15 M NaCl), PBS-gelatin (PBS containing 0.1% gelatin and 0.1% NaN_3), and DCC (2.5 g of Norit A and 0.25 g of Dextran T-70 in 1 L of PBS) were used.

Synthesis of Radioactive Photoaffinity Labeling Reagents. Δ^6-[1α,2α-$^3H_2$]T (∼1.4 Ci/mmol) was prepared from a mixture of [1α,2α-$^3H_2$]T (1 mCi, 5.4 μg, 18.7 nmol) and radioinert T (200 μg, 694 nmol), as previously described (*21*) by acid-catalyzed dehydrogenation of the corresponding 17-acetate derivative with dichlorodicyanobenzoquinone (*30*). The product, controlled by reverse-phase HPLC on a C_{18} column (Nucleosil 5 μm, 0.46 × 15 cm) with an aqueous acetonitrile gradient (40−100% CH_3CN in 45 min), showed a radiochemical purity greater than 98%.

Purification of Rabbit SHBG. Rabbit SHBG was isolated from a pool of rabbit serum obtained from a local farm. The concentration of SHBG in the pool (130 nM) was determined by a [^{3}H]DHT-binding capacity assay using Concanavalin A-Sepharose (*31*). SHBG was purified from rabbit serum in two steps according to the procedure previously described for the purification of human SHBG (*21*). The immunopurification step was performed using a monoclonal antibody against human SHBG cross-reacting with rabbit SHBG (Grenot & Cuilleron, unpublished results) and was followed by anion-exchange HPLC on a DEAE column (HEMA-IEC 1000 DEAE, Alltech). These two purification steps gave an overall yield of 70% determined by a [^{3}H]DHT-binding capacity assay using DCC separation (*32*). Purified SHBG was stored at −20 °C in PBS at a concentration of 2 mg/mL (23.3 μM), measured by UV absorption at 280 nm using the reported values of ϵ = 127 000 M^{-1} cm^{-1} and MW = 85.8 kDa, for rabbit SHBG (*8*).

Purification of Sheep SHBG. Sheep SHBG was isolated from a pool of sheep serum obtained from the local slaughterhouse. The concentration of SHBG in the pool (20 nM) was determined by the DHT-binding assay (*32*). Sheep SHBG did not cross-react with any of the monoclonal anti-human SHBG antibodies available in the laboratory and was purified by affinity chromatography using an immobilized DHT derivative. The affinity column was prepared according to a previously reported procedure (*33*). The activated *N*-hydroxysuccinimide ester of 17α-hemiglutaramido-17β-hydroxyandrostan-3-one (*34*) was coupled to bovine serum albumin (BSA). The steroid−BSA conjugate was purified by gel filtration on Sepharose CL 6B to eliminate noncovalently coupled steroid and immobilized on Sepharose 4B. The sheep serum (2 L) was incubated batchwise with the DHT-BSA-Sepharose affinity gel (75 mL) overnight at 4 °C with mild agitation. After filtration of the unretained fraction, the gel was washed several times in batch at 4 °C, with 200 mL of PBS buffer containing 0.2% of Tween 20 until no protein content could be detected by UV absorbance at 220 nm. The affinity resin was then equilibrated with 300 mL of 20 mM Tris-HCl buffer, pH 7.4, containing 0.5 M KCl and 10% of dimethylformamide. Retained SHBG was eluted by three successive incubations for 2 h at 4 °C with 100 μM of DHT in 100 mL of the equilibration buffer. The

purified DHT-bound SHBG fractions were extensively dialyzed against distilled water and lyophilized. The lyophilized fraction was dissolved in 3 mL of water, dialyzed twice against 1 L of 5 mM phosphate buffer, pH 7.4, and further purified by anion-exchange HPLC as described above for rabbit SHBG. These two purification steps gave purified steroid-free sheep SHBG in about 50% overall yield determined by the DHT-binding capacity assay (*32*). Purified SHBG was stored at −20 °C in PBS at a concentration of 2 mg/mL (21.4 μM) measured by UV absorption at 280 nm, using the reported values of $\epsilon = 114\,000\ M^{-1}\ cm^{-1}$ and MW = 93.4 kDa for human SHBG (*35*), and by the BCA protein assay.

Binding Measurements. The association constants of purified rabbit and sheep SHBGs with DHT, T, E_2, and Δ^6-T were determined by the method of Scatchard (*36*) using equilibrium dialysis. Aliquots (1 mL) of a solution of purified SHBG (15 nM) in PBS−gelatin buffer were placed inside dialysis bags and dialyzed for 20 h at 25 °C against eight concentrations (ranging from 4 to 80 nM) of tritiated steroid (the specific activity of tritiated steroid was adjusted to 1.4 Ci/mmol with radioinert steroid), placed outside the bag in 10 mL of PBS buffer.

The rates of dissociation of [^{3}H]DHT from purified rabbit and sheep SHBGs were determined by an incubation of equimolar concentrations (10 nM) of purified protein and [^{3}H]DHT in 200 μL of PBS−gelatin buffer for 1 h at 4 °C followed by the addition of an excess of radioinert DHT (1 μM). At 1, 2, 5, 10, 15, 20, and 30 min intervals after the addition of radioinert DHT, 1 mL of concentrated DCC (10 g of Norit A and 1 g of Dextran T-70 in 1 L of PBS) was added to each of the corresponding incubation mixtures and the amount of residual [^{3}H]DHT bound to SHBG was counted.

Photoaffinity Labeling. The conditions of photoaffinity labeling were essentially those previously described (*21*). Purified SHBG was first incubated for 1 h at 22 °C and for 15 min at 4 °C, under an argon atmosphere, in the dark, with either the radioinert (photoinactivation) or the tritiated Δ^6-T photoreagent, and irradiated for 30 min at 4 °C under an argon atmosphere at $\lambda > 300$ nm, using a 2 mm thick Pyrex filter (*37*). After irradiation, the dissociation of noncovalently bound steroid was performed by exclusion chromatography of the irradiation mixture on microcolumns (Trisacryl GF 05, 0.3 × 12 cm) equilibrated with 0.25 M Tris-HCl, pH 8.5, containing 6 M guanidinium chloride.

The following stoichiometries were employed in the incubation step prior to irradiation. (1) Photoinactivation experiments: SHBG (0.1 nmol) was incubated with Δ^6-T (1 nmol) in the absence or in the presence of DHT (10 nmol) in 100 μL of PBS−gelatin buffer. (2) Kinetics of covalent attachment: SHBG (0.1 nmol) was incubated with Δ^6-[1α,2α-$^3H_2$]T (0.3 nmol) in the absence or in the presence of DHT (30 nmol) in 100 μL of PBS−gelatin buffer. (3) Determination of HPLC profiles of tryptic digests: (a) SHBG (2 nmol) was incubated with Δ^6-T (6 nmol) containing Δ^6-[1α,2α-$^3H_2$]T (3×10^5 cpm) in 200 μL of PBS (without gelatin) in the absence or in the presence of DHT (60 nmol); (b) heat-denatured SHBG (2 nmol) was incubated with Δ^6-T (6 nmol) containing Δ^6-[1α,2α-$^3H_2$]T (3×10^5 cpm) in 200 μL of PBS. (4) Sequence analysis: (a) rabbit SHBG (24 nmol) was incubated with Δ^6-T (72 nmol) containing Δ^6-[1α,2α-$^3H_2$]T (9×10^6 cpm) in 1 mL of PBS; (b) sheep SHBG (50 nmol) was incubated with Δ^6-T (150 nmol) containing Δ^6-[1α,2α-$^3H_2$]T (50×10^6 cpm) in 4 mL of PBS. (5) Characterization of the photolabeled tryptic peptides by enzymatic subcleavage and mass spectrometry: rabbit and sheep SHBGs (150 nmol) were incubated with Δ^6-T (600 nmol) containing Δ^6-[1α,2α-$^3H_2$]T (200×10^6 cpm) in 7.5 mL of PBS.

Gel Electrophoresis. Sodium dodecyl sulfate ($NaDodSO_4$)−polyacrylamide gel electrophoresis (*38*) was performed in a vertical slab-gel apparatus. Unlabeled SHBG samples were stained with silver nitrate (*39*) whereas photoaffinity-labeled SHBGs were detected by fluorography, after treatment of the gel with 1 M sodium salicylate, using a Kodak X-OMAT film. Recombinant molecular weight standard mixture (15−150 kDa) was purchased from Sigma.

Tryptic Cleavage of Photolabeled SHBG and Purification of Photolabeled Fragments. Photolabeled SHBG samples were reduced with dithioerythritol, carboxymethylated by iodoacetic acid, and transferred in 0.5 M Tris-HCl, pH 8.2 buffer by exclusion chromatography on Trisacryl GF 05. Cleavage with TPCK-trypsin (2% enzyme/SHBG ratio, w/w) was accomplished by incubation for 24 h at 37 °C. Trypsin hydrolysates were separated by reverse-phase HPLC using an aqueous acetonitrile gradient in the presence of 0.1% TFA. The preparative separation of labeled peptides was achieved by three or four successive HPLC purifications in the presence of 0.1% TFA in the elution gradient, for the first, third, and fourth separations, and in the presence of 20 mM NH_4HCO_3, pH 7.5, in the aqueous fraction of the gradient for the second separation.

Enzymatic Subcleavage of the Photolabeled Tryptic Peptide of Rabbit SHBG. The fractions of the radioactive peak from the second HPLC step (in neutral conditions) were concentrated to 400 μL, under a nitrogen stream in order to eliminate acetonitrile. One-half of this concentrated fraction was incubated with elastase (10 μg) for 20 h at 37 °C after addition of 50 mL of 1 M Tris-HCl, pH 8.5, and the other half was incubated directly with carboxypeptidase B (20 μg) for 20 h at 37 °C. The cleaved radioactive peptides were purified by reverse-phase HPLC on a C_{18} column using an aqueous acetonitrile gradient in the presence of 0.1% TFA.

Deglycosylation of the Photolabeled Tryptic Peptide S_1 of Sheep SHBG. The fractions of the radioactive peptide S_1 collected at the second HPLC step (in neutral conditions) were concentrated to 400 μL under a nitrogen stream to eliminate acetonitrile and incubated with *N*-glycosidase F (10 μg) for 20 h at 37 °C. The deglycosylated radioactive peptide was purified by reverse-phase HPLC on a C_{18} column using an aqueous acetonitrile gradient in the presence of 0.1% of TFA.

Edman Sequence Determinations. Automated Edman sequencing of purified peptides was performed in a gas-phase sequencer equipped with an on-line phenylthiohydantoin (PTH) analyzer (Applied Biosystems, models 470 and 473 sequencers).

Mass Spectrometry. The electrospray mass spectra of the purified photolabeled peptides (200−500 pmol) were recorded with a Hewlett-Packard 5989 mass spectrometer or a VG Platform (Micromass) mass spectrometer, equipped with single quadrupole and an atmospheric pressure elec-

Table 1: Association Constants of Purified Rabbit and Sheep SHBGs with DHT, T, and E_2 and Half-Dissociation Times of DHT

	K_a (10^9 M^{-1}) at 22 °C				
SHBG	DHT	T	Δ^6-T	E_2	$t_{1/2}$ (min)
rabbit	0.4	0.2	0.16	nd[a]	9
sheep	1.2	0.8	0.65	0.2	25

[a] The affinity of rabbit SHBG for E_2 was too low to allow the determination of a precise association constant from the slope of the Scatchard plot.

trospray ion source at a resolution of 1000 (50% valley). The peptides were injected in 60 μL of 50% aqueous methanol containing 1% acetic acid at a flow rate of 2 or 10 μL/min. The quadrupoles were scanning from *m*/*z* 200 to 2200. The positions of molecular ion peaks were estimated to be accurate to $\pm$ 0.2 *m*/*z* units. The measured masses derived from $[M + nH]^{n+}$ multicharged ions were estimated to be accurate to $\pm n \times 0.2$ *m*/*z* units, and were compared with calculated chemical masses.

RESULTS

*Characterization of Purified Rabbit and Sheep SHBGs.*The absence of denaturation and the steroid-free character of purified rabbit and sheep SHBGs were demonstrated by the correlation of the concentration of steroid-binding sites (21 and 18 nmol/mL for rabbit and sheep SHBGs, respectively) estimated from Scatchard plots (*36*) established after equilibrium dialysis experiments, with the concentration of SHBG homodimer estimated either from UV absorption at 280 nm (23.3 and 21.4 nmol/mL) or from a protein assay (21.4 nmol/mL) for sheep SHBG only.

The purities of rabbit and sheep SHBGs were controlled by Edman sequencing of 14 amino acid residues of the N-terminal part of each protein. Edman sequencing of rabbit SHBG showed equal amounts of two N-terminal sequences, one corresponding to T Q R A Q D S P A V H L I N as previously reported (*8*, *9*) and the other to the same peptide deprived of the first two residues. Similarly, Edman sequencing of sheep SHBG showed equal amounts of the full-length sequence A P A L R P P L P S Q T T E and of its subcleaved form deprived of the first two N-terminal amino acids. These two N-terminal sequences of sheep SHBG are in agreement with that established by cDNA sequencing but begin, respectively, three amino acids and one amino acid before the L residue predicted as the probable N-terminal amino acid (*10*). Heterogeneity of the location of the N-terminal amino acid has been previously reported for human and rabbit SHBGs (*4*, *8*).

The association constant for DHT binding to immunopurified rabbit SHBG at 22 °C estimated from Scatchard plots (0.4×10^9 M^{-1}) was higher than that measured for T (0.2×10^9 M^{-1}), as previously reported for the binding of DHT and T to unpurified or purified rabbit SHBG (*16*, *17*, *40*), whereas the association constant for the binding of E_2 was too low to be determined accurately from the Scatchard plot (Table 1). The association constants measured for DHT and T binding to purified sheep SHBG (1.2 and 0.8×10^9 M^{-1}) were higher than those of rabbit SHBG. The association constant for E_2 binding to purified sheep SHBG (0.2×10^9 M^{-1}) revealed a significant binding affinity for E_2 that represented about 20% of those measured for DHT or T.

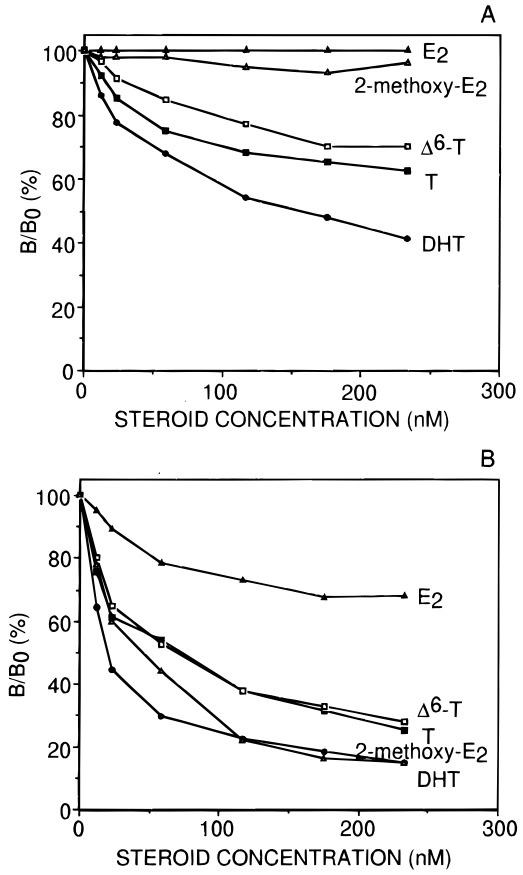

FIGURE 1: Competitive binding assays for DHT, T, Δ^6-T, E_2, and 2-methoxyestradiol with purified SHBGs. Aliquots (100 μL) of purified rabbit SHBG (2.0 nM in PBS-gelatin) were incubated with [^{3}H]DHT (50 000 cpm, 4.5×10^{-4} nmol) and seven concentrations (ranging from 10 to 240 nM) of DHT and of each of the radioinert T, Δ^6-T, E_2, and 2-methoxyestradiol competitors in 400 μL of PBS-gelatin, for 1 h at 22 °C and for 15 min at 4 °C. Free and bound steroids were separated by DCC. For each competitor concentration, the radioactivity of bound [^{3}H]DHT (*B*) is expressed as percent of radioactivity bound in the absence of competitor (B_0): DHT (●), T (■), Δ^6-T (□), E_2 (▲), 2-methoxyestradiol (△), immunopurified rabbit SHBG (A); purified sheep SHBG (B).

This contrasts with the much lower relative binding affinity for E_2 reported for unpurified SHBG from ram plasma (*19*), due possibly to effects of serum albumin, and indicates that sheep SHBG shares with human SHBG the ability to recognize both androgens and estrogens although the binding affinities are lower. Purified rabbit and sheep SHBGs showed also significantly different half-dissociation times for DHT (9 and 25 min, respectively) which are in agreement with the differences in their association constants for DHT (Table 1).

The experiments of displacement of [^{3}H]DHT bound to immunopurified rabbit SHBG by E_2, as well as by 2-methoxyestradiol, reported as a derivative having a higher binding affinity for human SHBG than E_2 (*41*), showed very weak competitive effects of these steroids in the concentration range studied (Figure 1A) as expected from the very low estrogen-binding affinity of rabbit SHBG. On the other hand, [^{3}H]DHT bound to purified sheep SHBG was significantly displaced by E_2 whereas a much higher competitive effect of 2-methoxyestradiol, almost similar to that of DHT, was observed (Figure 1B), as previously described for human

Table 2: Photoinactivation of Rabbit and Sheep SHBGs by Radioinert Δ^6-T

SHBG	Inactivation of initial binding capacity (%)[a]	
	Δ^6-T	Δ^6-T + DHT
rabbit SHBG	65	18
sheep SHBG	59	21

[a] Photoinactivation percentages determined from the [^{3}H]DHT binding capacity of photoinactivated SHBG (0.1 nmol) after removal of noncovalently bound Δ^6-T by treatment with DCC (1 mL, 1 h at 20 °C).

SHBG (*41*), thus confirming the ability of sheep SHBG to recognize both androgens and estrogens.

The association constants for Δ^6-T binding to purified rabbit and sheep SHBGs (0.16 and 0.65 $\times$ 10^9 M^{-1}) were only slightly lower than those measured for T (Table 1) as reported for human SHBG (*21*). For both proteins, the abcissa intercepts of the linear Scatchard plots for the binding of Δ^6-T, DHT, T, and E_2 were the same within experimental error, thus showing the binding of all these derivatives at the same site.

The experiments of displacement of [^{3}H]DHT bound to rabbit and sheep SHBGs by Δ^6-T and T confirmed that the presence of a Δ^6 double bond leads to a slightly decreased cross-reactivity when compared to testosterone (Figure 1A,B).

Kinetics of Photoinactivation. The extent of photoinactivation of rabbit and sheep SHBGs by radioinert Δ^6-T was determined after irradiation for 30 min at $\lambda > 300$ nm of each sample of SHBG using a 10:1 steroid/protein molar ratio, in the absence of DHT for the measurement of total inactivation, and in the presence of a 100-fold molar excess of DHT for the measurement of nonspecific inactivation. Photoinactivation of rabbit SHBG led to inactivation of 65% of the initial binding capacity for [^{3}H]DHT including 18% of nonspecific inactivation (Table 2), whereas photoinactivation of sheep SHBG in the same conditions led to 59% and 21% of total and nonspecific photoinactivations, respectively. The percentages of specific photoinactivation of rabbit and sheep SHBGs (47% and 38%) were lower than that of the 65% measured in the same conditions for human SHBG (*21*).

Irradiation for 30 min at $\lambda > 300$ nm of rabbit and sheep SHBGs incubated with a 10-fold molar excess of DHT, followed by elimination of noncovalently bound steroid with DCC, led to the recovery of 100% of the initial binding capacity of the two proteins, thus demonstrating the stability of both proteins to irradiation at $\lambda > 300$ nm (data not shown) as previously reported for human SHBG (*21*).

Kinetics of Covalent Photoattachment of Δ^6-[^{3}H]T to Rabbit and Sheep SHBGs. The covalent photoattachment of Δ^6-[^{3}H]T to rabbit and sheep SHBGs was studied at $\lambda >$ 300 nm, using a 3:1 steroid/protein molar ratio in the absence or in the presence of a 30-fold molar excess of DHT. In both cases, the time course of photolabeling was parallel with that of photoinactivation and maximal levels of covalently attached radioactivity were reached after 30 min irradiation, as reported for human SHBG (*21*). The highest levels of specific attachment of Δ^6-[^{3}H]T (0.33 and 0.30 mol of label/mol of rabbit and sheep SHBG, respectively) were similar for the two SHBGs despite their differences in steroid-binding properties. Increasing the amount of steroid from a 3:1 up to a 10:1 ratio of steroid/protein concentrations, or the irradiation time, did not augment significantly the amounts of specific labeling which remained in both cases lower than that found for human SHBG (0.50 mol of label/mol of protein) (*21*).

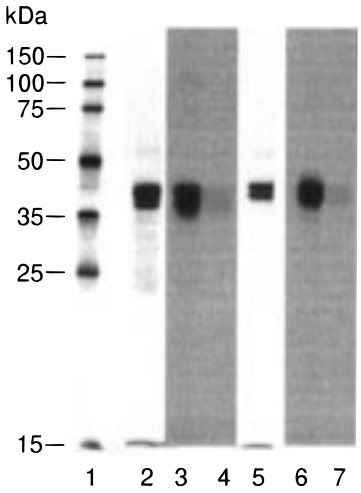

FIGURE 2: $NaDodSO_4$−polyacrylamide gel electrophoresis of unlabeled and photolabeled samples of purified rabbit and sheep SHBGs: molecular weight markers (lane 1), unlabeled rabbit SHBG stained with $AgNO_3$ (lane 2), rabbit SHBG photolabeled with Δ^6-[^{3}H]T in the absence and in the presence of DHT, revealed by fluorography (lanes 3 and 4), unlabeled sheep SHBG stained with $AgNO_3$ (lane 5), and sheep SHBG photolabeled with Δ^6-[^{3}H]T in the absence and in the presence of DHT, revealed by fluorography (lanes 6 and 7).

Gel Electrophoresis of Photolabeled Rabbit and Sheep SHBGs. The radioactivity distribution after $NaDodSO_4$−polyacrylamide gel electrophoresis of photolabeled rabbit and sheep SHBGs was found exclusively at the level of the characteristic bands of pure unlabeled SHBGs (Figure 2). The specificity of photolabeling is shown by the large decrease of the radioactivity observed in the samples photolabeled in the presence of DHT. The absence of any significant effects on the electrophoretic mobility of SHBG monomers indicates that photoaffinity labeling with the Δ^6-T photoreagent induced no modification of the charge and produced no protein cleavage.

Tryptic Cleavage of Photolabeled SHBGs. Rabbit and sheep SHBGs covalently photolabeled with Δ^6-[^{3}H]T in the absence or in the presence of an excess of DHT were reduced, carboxymethylated, and digested with trypsin. The tryptic digests were fractionated by reverse-phase HPLC on a C_{18} column using an aqueous acetonitrile gradient in acidic conditions (0.1% TFA).

The radioactivity profile of the tryptic digest of photolabeled rabbit SHBG showed a single radioactive peak which was almost totally abolished when a 30-fold molar excess of DHT was added to the Δ^6-[^{3}H]T photoreagent (Figure 3). A control experiment was made on tryptic fragments of heat-denatured SHBG (1 h at 60 °C), irradiated in the presence of Δ^6-[^{3}H]T, which showed a low radioactive peak similar to the peak of nonspecific labeling observed in the sample photolabeled in the presence of DHT (data not shown).

The radioactivity profiles of the tryptic digest of sheep SHBG revealed the presence of two major radioactive peaks

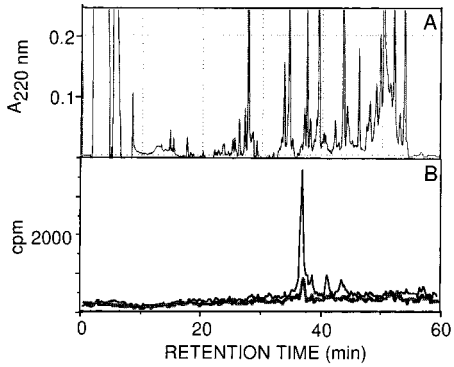

FIGURE 3: Reverse-phase HPLC separation of tryptic peptides of rabbit SHBG photolabeled with Δ^6-[^{3}H]T. The tryptic digests were applied on a C_{18} column (Nucleosil 5 μm, 0.46 × 15 cm) and eluted at 1 mL/min using an aqueous acetonitrile gradient in the presence of 0.1% TFA (0−10% CH_3CN in 5 min and 10−50% CH_3CN in 45 min). Peptide elution was monitored at 220 nm, and radioactivity profiles were determined on-line with a Flo-one Packard radiodetector. (A) UV absorbance detection of tryptic digest of rabbit SHBG photolabeled with Δ^6-[^{3}H]T; (B) radioactivity detection of tryptic digests of rabbit SHBG photolabeled with Δ^6-[^{3}H]T (solid line) alone, or in the presence of DHT (dark gray line).

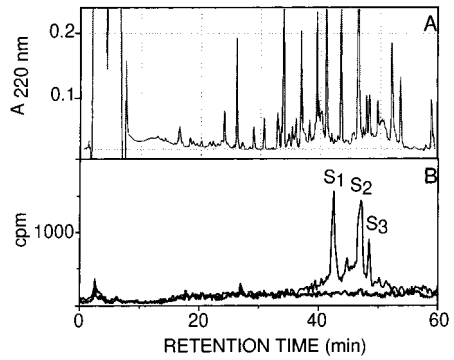

FIGURE 4: Reverse-phase HPLC separation of tryptic peptides of sheep SHBG photolabeled with Δ^6-[^{3}H]T. The tryptic digests were applied on a C_{18} column (Nucleosil 5 μm, 0.46 × 15 cm) and eluted at 1 mL/min using an aqueous acetonitrile gradient in the presence of 0.1% TFA (0−10% CH_3CN in 5 min and 10−50% CH_3CN in 45 min). Peptide elution was monitored at 220 nm, and radioactivity profiles were determined on-line with a Flo-one Packard radiodetector. (A) UV absorbance detection of tryptic digest of sheep SHBG photolabeled with Δ^6-[^{3}H]T; (B) radioactivity detection of tryptic digests of sheep SHBG photolabeled with Δ^6-[^{3}H]T (solid line) alone, or in the presence of DHT (dark gray line).

S_1 and S_2 along with a minor radioactive peak S_3, which were no longer detectable in the sample photolabeled in the presence of a 30-fold molar excess of DHT (Figure 4) and in a photolabeled heat-denatured sample (data not shown).

Purification of Photoaffinity-Labeled Peptides. The separation of the radioactive peptides from the tryptic digest of rabbit SHBG photolabeled with Δ^6-[^{3}H]T was made by three successive chromatographies on a reverse-phase C_{18} HPLC column using an aqueous acetonitrile gradient in acidic conditions (0.1% TFA) for the first and third purifications and in neutral conditions (20 mM NH_4HCO_3, pH 7.5) for the intermediate purification. This protocol of separation confirmed the presence of a single radioactive peptide peak which could be separated in about 2% yield in preparative experiments performed on 50 nmol of SHBG.

The preparative separation of the tryptic digest of sheep SHBG photolabeled with Δ^6-[^{3}H]T was made by chromatography on a reverse-phase C_{18} HPLC column using the aqueous acetonitrile gradient in acidic conditions (0.1% TFA) employed to establish the radioactivity profile of the tryptic digest (Figure 4). The radioactivity recovered under the major peaks S_1, S_2, and the minor peak S_3, corresponded respectively to 30%, 35%, and 8% of the radioactivity injected on the column. Peptidic peaks S_1 and S_2 were separated by three successive chromatographies, in neutral conditions (20 mM NH_4HCO_3, pH 7.5) for the first step and in acidic conditions (0.1% TFA) for the second and third steps. This protocol of purification led to the recovery of 0.2 nmol of each of the two purified peptidic fractions S_1 and S_2 from 50 nmol of photolabeled sheep SHBG.

On the other hand, the chromatography in neutral conditions of peak S_3 gave two radioactive peaks. The separation of each radioactive peak in acidic conditions showed that the major radioactive fraction was eluted at the retention time of the free photolyzed steroid and was not further studied whereas the second peak could not be purified to homogeneity, owing to the low amount of radioactivity.

Table 3: Amino Acid Sequence Analysis of Rabbit Tryptic Peptide Leu-118-Lys-134 Photolabeled with Δ^6-[^{3}H]T[a]

cycle	amino acid	PTH deriv (pmol)	radioactivity (dpm)
1	L	240.0	1272
2	S	45.0	964
3	Q	131.5	944
4	V	165.0	792
5	S	34.1	716
6	G	86.6	424
7	T	32.6	628
8	L	68.0	792
9	H	42.2	952
10	D	42.8	1225
11	K	45.8	1620
12	P	30.0	984
13	Q	32.9	588
14	P	8.9	492
15	V	14.3	412
16			340
17	K	9.5	404

[a] Δ^6-[3H]T-tryptic peptide: 56 000 dpm (400 pmol).

Characterization of the Photoaffinity-Labeled Tryptic Peptide of Rabbit SHBG. Amino acid sequence analysis of the purified photolabeled tryptic peptide of rabbit SHBG showed a single sequence in which no known amino acid was identified at cycle 16 (Table 3). This sequence corresponds to the peptide fragment Leu-118-Lys-134 of rabbit SHBG in which the missing amino acid at cycle 16 is Met-133. The picomoles of the PTH derivative of leucine measured at the first Edman cycle (240 pmol) were in agreement with the amount of peptide introduced in the sequencer, calculated from the specific activity of the photoreagent (400 pmol), and correspond to usual yields of automated Edman sequencing. The radioactivity recovered in the PTH effluents represented 24% of the radioactivity introduced on the sequencer but showed no prominent peak although a low increase was found at cycles 10−11 corresponding to Asp-127 and Lys-128 which were identified without concomitant decrease in yields of PTH derivatives. However, no other contaminating sequence could be detected, whereas only a low amount of radioactivity remained on the filter (3%), thus suggesting that the covalent fixation of Δ^6-[^{3}H]T to the tryptic peptide 118−134 is labile in the

conditions of Edman sequencing. On the other hand, the characterization of the PTH derivatives of all amino acids of the peptide, except for the Met-133 residue, suggests that Δ^6-[^{3}H]T was first covalently linked to this amino acid and then progressively released by partial hydrolysis at each Edman cycle.

The presence of Δ^6-T on the peptide fragment Leu-118-Lys-134 of rabbit SHBG was confirmed by mass spectrometry. The electrospray mass spectrum of the purified photolabeled tryptic peptide showed two major peaks at *m*/*z* 538.7 and 717.9 corresponding to the $[M + 4H]^{4+}$ and $[M + 3H]^{3+}$ molecular ions of an unprotonated peptide of measured mass of 2150.8 ± 0.8 and two minor peaks at *m*/*z* 542.9 and 723.5 corresponding to a measured mass of 2166.6 ± 0.8. The measured mass of 2150.8 is in agreement with the calculated mass of 2151.6 corresponding to the addition of one mole of Δ^6-T to the peptide. On the other hand, the measured mass of 2166.6 corresponds to an increment of 15.8 which may result from the presence of a contaminating sulfoxide derivative of Met-133.

To localize unambiguously the photolabeled amino acid, the purified photolabeled tryptic peptide Leu-118-Lys-134 of rabbit SHBG was subcleaved either by elastase or by carboxypeptidase B. Hydrolysis by elastase showed that the initial radioactivity was almost quantitatively recovered (88%) in a single radioactive peptide which was purified by reverse-phase HPLC and analyzed by mass spectrometry. The electrospray mass spectrum showed a major $[M + H]^+$ ion at *m*/*z* 564.7 ± 0.2, which corresponds to the mass of the protonated dipeptide Met-133-Lys-134 conjugated with one mole of Δ^6-T (calculated unprotonated mass 563.8). This demonstrates that Δ^6-T is present exclusively on the C-terminal dipeptide, most probably on the Met-133 amino acid residue which could not be identified by Edman sequencing of the photolabeled tryptic peptide precursor.

The exclusive photolabeling of Met-133 was confirmed by the mass spectrum of the photolabeled peptide obtained after cleavage of the photolabeled tryptic peptide Leu-118-Lys-134 at the C-terminal lysine by carboxypeptidase B. The hydrolysis with carboxypeptidase B and separation of the products by reverse-phase HPLC showed a major radioactive peak at a retention time consistent with the loss of the C-terminal Lys-134 residue and a minor peak of residual uncleaved peptide. The electrospray mass spectrum of the cleaved peptide showed two multicharged ions $[M + 3H]^{3+}$ and $[M + 2H]^{2+}$ at *m*/*z* 675.5 and 1012.7, corresponding to a measured mass of 2023.3 ± 0.6, in agreement with the mass of 2023.4 calculated for the addition of one mole of Δ^6-T to the peptide Leu-118-Met-133. The recovery of almost all of the initial radioactivity of the photolabeled peptide Leu-118-Lys-134 either in the C-terminal dipeptide Met-133-Lys-134 or in the subcleaved peptide Leu-118-Met-133 and the characterization by mass spectrometry of the addition of one molar equivalent of Δ^6-T photoreagent on these three peptides confirmed unambiguously that Δ^6-T is attached exclusively on Met-133, which is the only amino acid residue common to the two radioactive subcleaved peptides.

Characterization of the Photoaffinity-Labeled Tryptic Peptide of Sheep SHBG. Edman sequencing of the two major photolabeled tryptic peptide fractions S_1 and S_2 of sheep SHBG showed the same single sequence identified as the peptide fragment Gln-126-Arg-140 which contained no known PTH derivative at cycle 14 corresponding to Met-139. Moreover, the photolabeled peptide S_1 showed another unidentifiable PTH derivative at cycle 8 corresponding to the Asn-133 amino acid residue located in a consensus site of N-glycosylation Asn-Asn-Ser (Table 4). The picomoles of the PTH derivatives of glutamine measured at the first Edman cycle were in both cases in agreement with the amounts of peptide introduced in the sequencer, calculated from the specific activity of the photoreagent. The radioactivity profile of the PTH effluents of peptide S_1 showed a major peak of radioactivity eluted at cycle 14 corresponding to the unidentified Met-139 residue. This major peak represented 19% of the radioactivity recovered in PTH effluents but only 9.8% of the radioactivity introduced on the sequencer. A smaller peak of radioactivity was also found at cycle 11, which was attributed to a release of the radioactive steroid by hydrolysis in the conditions of Edman sequencing rather than to the partial labeling of Gln-136 which was quantitatively recovered as PTH derivative. On the other hand, no radioactive peak was present at cycle 8 corresponding to the unidentified Asn-133 residue. The picomoles of the missing methionine residue (7 pmol), estimated from the radioactivity eluted at cycle 14, were much lower than the amount measured for the preceding isoleucine (31.2 pmol) but confirmed that Met-139 is an important site of photolabeling by the Δ^6-T photoreagent, whereas the partial release of radioactivity at the other cycles prevented any further localization of the photoreagent. By contrast, the radioactivity profile of PTH effluents of peptide S_2 revealed a pattern of release of radioactivity without any detectable peak, even at cycle 14, as found above for the photolabeled peptide from rabbit SHBG. The absence of detectable contaminating sequences and the low amount of radioactivity remaining on the filter (1%) eliminated the possibility that another photolabeled peptide was copurified with the tryptic peptide 126−140. Therefore, the detection of PTH derivatives of all amino acids of peptide S_2 in usual yields, except for the undetected Met-139 residue, strongly supports the conclusion that Met-139 is a site of photola-

Table 4: Amino Acid Sequence Analysis of Sheep Tryptic Peptides Gln-126-Arg-140 Photolabeled with Δ^6-[^{3}H]T

	Δ^6-[3H]T-tryptic peptide S_1 100 000 dpm (72 pmol)			Δ^6-[3H]T-tryptic peptide S_2 45 000 dpm (25 pmol)	
cycle	amino acid	PTH (pmol)	radioactivity (dpm)	amino acid	PTH pmol	radioactivity (dpm)
1	Q	66.1	2216	Q	11	1172
2	V	60.5	2714	V	9.1	1268
3	F	55.3	1937	F	9.1	1032
4	G	53.0	1972	G	7.1	900
5	Q	56.7	1357	Q	4.5	724
6	L	52.0	1960	L	5.2	728
7	A	42.9	1705	A	5.4	608
8			1984	N	3.1	704
9	N	63.0	2308	N	*a*	908
10	S	13.7	4489	S	1.1	1008
11	Q	41.2	6009	Q	1.7	924
12	L	24.7	4395	L	1.7	872
13	I	31.2	4547	I	2.0	672
14		(7)[b]	9767			712
15	R	11	3538	R	0.45	480

[a] Residue identified but not quantified. [b] Picomoles of amino acid estimated from the radioactivity eluted at cycle 14 of Edman sequencing.

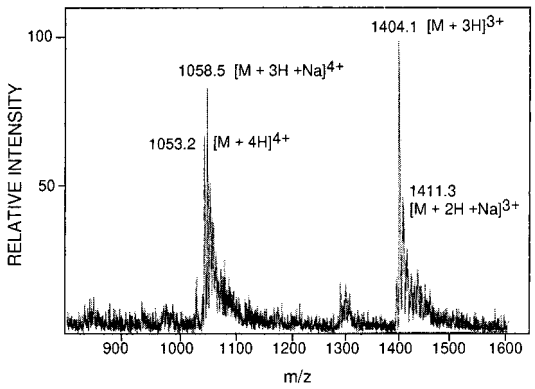

FIGURE 5: Electrospray mass spectrum of peptide S_1 of sheep SHBG photolabeled with Δ^6-[^{3}H]T.

beling. All these findings suggest that photolabeling of Met-139 of sheep SHBG is heterogeneous toward acid hydrolysis, the most resistant fraction being present on peptide S_1.

The presence of the consensus site of N-glycosylation Asn-Asn-Ser 133−135 on the peptide fragment Gln-126-Arg-140 of sheep SHBG and the absence of detection of the PTH derivative of Asn-133 in photolabeled peptide S_1 but not in photolabeled peptide S_2 both point to the hypothesis that only peptide S_1 is glycosylated. The electrospray mass spectrum of the peptide S_1 showed two main peaks at *m/z* 1053.2 and 1404.1 corresponding to the $[M + 4H]^{4+}$ and $[M + 3H]^{3+}$ ions of a peptide of measured mass of 4208.9 ± 0.8 in accordance with the presence of a N-linked oligosaccharide chain at Asn-133 (Figure 5). Smaller peaks of higher masses were also found, which correspond probably to Na^+ and K^+ adducts. To characterize unequivocally the presence of the photoadduct, the photolabeled peptide S_1 was treated by N-glycosidase to give a less polar radioactive peak S'_1 which was eluted from the reverse-phase HPLC column at a retention time identical to that of the photolabeled peptide S_2. The electrospray mass spectrum of the deglycosylated peptide S'_1 showed two peaks at *m/z* 669.5 and 1003.7 corresponding to the $[M + 3H]^{3+}$ and $[M + 2H]^{2+}$ ions of a peptide of measured mass of 2005.4 ± 1.2, which is in agreement with the mass of 2006.4 calculated for the addition of one mole of Δ^6-T to the deglycosylated peptide. The accuracy of mass measurement for peptide S'_1 was lower than for the other peptides owing to lower signal/noise ratios. On the other hand, the difference of mass of 2203.5 between glycosylated peptide S_1 and deglycosylated peptide S'_1 corresponds to the mass of a disialylated biantennary glycan without fucose residue (*42*, *43*), as reported for rabbit and human SHBGs (*44*).

DISCUSSION

The photolabeling with Δ^6-T of Met-133 of rabbit SHBG and Met-139 of sheep SHBG, both homologous to Met-139 of human SHBG, confirms our previous results of photolabeling of the Met-139 of human SHBG with the same photoreagent (*21*) and suggests that this residue has a critical role in the interaction of all three proteins with steroid ligands. It should be noted that steroid dienone photoreagents have been found to react also with methionine residues in the case of steroid receptors (cf. ref *21*).

The covalent photoattachment of the Δ^6-T to Met-133 residue of rabbit SHBG was found labile in the acidic conditions of Edman sequencing, in contrast to the more stable photolabeling of the homologous Met-139 residue of human SHBG with the same photoreagent (*21*). Therefore, the photolabeling of Met-133 was confirmed by electrospray mass spectrometry of two subcleaved overlapping peptide fragments Leu-118-Met-133 and Met-133-Lys-134, each containing most of the photolabel initially present on the uncleaved tryptic peptide precursor. The identification of the photolabel on the dipeptide Met-133-Lys-134 having a size too small for maintaining any significant steroid-binding properties is a strong argument in favor of a covalent attachment since it rules out the possibility that the peaks observed by electrospray mass spectrometry may correspond to noncovalent interactions (*45*).

The stability of the covalent photoattachment of the Δ^6-T to Met-139 residue of sheep SHBG in the acidic conditions of Edman sequencing was found to depend on the partial glycosylation at the Asn-133 residue which led to two photolabeled tryptic peptides corresponding respectively to glycosylated and unglycosylated forms S_1 and S_2 of the peptide Gln-126-Arg-140. Only the radioactive photolabel covalently attached to the glycosylated peptide S_1 was stable in the acidic conditions of Edman sequencing. Mass spectrometry of the deglycosylated peptide S_1 confirmed the covalent fixation of one mole of Δ^6-T to this peptide, thus establishing unambiguously that glycosylation at Asn-133, in the proximity of the steroid-binding pocket, does not prevent the photoattachment of Δ^6-T to the binding site. However, no interpretable mass spectra could be obtained for the homologous unglycosylated peptide S_2.

The glycosylation at Asn-133 of sheep SHBG suggests that this residue is located near the surface of the SHBG molecule. The glycosylated Asn-133 residue is contiguous to the peptide segment Asn-134-Pro-149 homologous to the corresponding fragment of human SHBG containing the two known affinity-labeled amino acid residues Lys-134 (*22*) and Met-139 (*21*) assumed to interact respectively with opposite C-17 and C-3 to C-7 positions of steroid ligands, thus suggesting that residues Asn-134 of sheep SHBG and Lys-134 of human or rabbit SHBGs (human SHBG numbering) are both located at the surface of the protein. Such an assumption, valid only for the one of the two Lys-134 of SHBG homodimer which can be alkylated by 17β-bromoacetoxy-DHT (*22*), is in good agreement with the presence of an open side of the steroid-binding site in the vicinity of the 17β-OH group of the steroid ligand, as suggested earlier to explain the relatively high binding affinity of SHBGs of different species for 17α-substituted DHT ligands (*46*), and was confirmed in this study by the efficient affinity purification of sheep SHBG with an immobilized 17α-hemiglutaramidomethyl derivative of DHT. Therefore, the 17β-OH group of steroid ligands, known as a major structural determinant for high binding affinity to human SHBG, which has been postulated to interact directly through hydrogen bonding with the Lys-134 amino side chain of human or rabbit SHBG (*46*), may interact similarly with the homologous Asn-134 residue of sheep SHBG, as well as with His-134 of murine ABP/SHBG. This hypothesis is in keeping with the finding that the substitution of Lys-134 by His-134 in a recombinant human SHBG single mutant had no important effects on binding properties for androgens (*24*), whereas conversion to the neutral residue Ala-134 strongly

decreased the affinity for DHT (*25*). The SHBG molecule has also been reported to recognize other steroid ligands with substituents at the C-1 and C-2 positions, thus indicating that another open side of steroid-binding site may also exist in the proximity of these two positions (cf. ref *46*). All of these observations point to the hypothesis of a steroid-binding pocket lying near the surface of the SHBG molecule in which the Met-139 presumed to be present in the vicinity of the nonpolar C-3 to C-7 side of the steroid ligand should be located in a more buried hydrophobic part of the site, as suggested also by its proximity to the hydrophobic peptide fragment 141−148. Although these findings indicate that the steroid-binding site of SHBG may contain simultaneously hydrophobic and solvent-exposed regions, located respectively in the vicinity of the conserved and nonconserved residues homologous to Met-139 and Lys-134 of human SHBG, which are separated by a distance compatible for an interaction with the steroid ligand within a same subunit, the unambiguous assignment of their respective locations in the dimeric model must await the results of further experiments aimed at determining the relative positions and the distribution in the two interacting domains of the different pairs of affinity-labeled and unlabeled residues.

A model for the structure of the SHBG homodimer has been recently proposed, in which the steroid-binding site is postulated to lie at the interface of the N-terminal ends of the two linearly associated monomers (*47*). In this model, the possibility exists that the relatively well conserved hydrophobic peptide fragment 141−148 (human SHBG numbering), previously identified as a putative dimerization domain (*28*), may play a role. This domain lies in close proximity to the Met-139 residue shown in this work to remain in all cases a conserved site of interaction with the steroid photoreagent and could be expected to dimerize with two possible relative orientations. Since only one mole of steroid ligand can be bound per mole of SHBG homodimer one may speculate whether the assembly of two similarly oriented domains may have the potential advantage to induce mutual steric hindrance in the vicinity of the two Met-139 residues, which could contribute to limit the interaction at this level to a single steroid ligand per dimer, or may be totally avoided owing to steric repulsion.

The differences in the stabilities of the covalent bonds formed between Δ^6-T and human, rabbit, and unglycosylated and glycosylated sheep SHBGs in Edman sequencing conditions suggest a structural heterogeneity of the steroid-methionine photoadducts. The stereochemistry and the mechanism of photolabeling could be influenced by interspecies differences in the positioning of the steroid photoreagent toward the amino acid side chain in the steroid-binding sites, presumably related in part to the effects of the nonconserved peptide fragments homologous to region Pro-130-Ile-138 of human SHBG (*28*−*29*), including possible modifications caused by the glycosylation of Asn-133 of sheep SHBG. Indirect evidence for elucidating partially the structure of the photolabeled Met-133 residue of rabbit SHBG was provided by the observation that this photolabeled amino acid could be cleaved easily either at its N-terminal amide bond by elastase or at its C-terminal one by carboxypeptidase B, thus suggesting that the photolabeling with Δ^6-T might occur on the methionine side-chain rather than on the atoms forming the peptidic chain, since in this latter case steric hindrance caused by the photolabel could be expected to inhibit, at least partially, one or both of the two enzymatic cleavages. In the absence of more detailed structural analysis of the photoadducts of Δ^6-T with methionine for the different SHBGs, one can only speculate about the photochemical mechanisms, which may explain differences in their acid sensitivity. A radical mechanism of photoattachment through carbon−carbon bonds (*21, 48*) is expected to favor the formation of products resistant to acid hydrolysis although coupling with the terminal thiomethyl group might allow the release of the photoattached steroid by acid cleavage of the thioether bond. On the other hand, photoattachment through carbon−heteroatom bonds may lead to more acid-labile products such as hemithioacetal adducts known as thiol protective groups (*49*). In both cases, the sensitivity to acid hydrolysis may also be dependent on the relative positions of residual unsaturated or carbonyl bonds on the steroid and on the possible presence of photorearranged products due to the relatively long irradiation times employed for improving the yields of covalent attachment. However, the hypothesis of a protecting effect of the N-glycosylated side-chain at Asn-133 of sheep SHBG against acid hydrolysis cannot be ruled out, owing to the large size of the oligosaccharide. Further studies will be required to determine the structural bases of these heterogeneities and their role in binding specificities.

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SUPPORTING INFORMATION AVAILABLE

Figures showing the electrospray mass spectrum of the photolabeled tryptic peptide 118−134 of rabbit SHBG, of the photolabeled dipeptide Met-Lys 133−134 of rabbit SHBG prepared by subcleavage of peptide 118−134 with porcine elastase, of photolabeled peptide 118−133 of rabbit SHBG prepared by subcleavage of peptide 118−134 with carboxypeptidase B, and of deglycosylated photolabeled tryptic peptide 126−140 (peptide S′1) of sheep SHBG (4 pages). Ordering information is given on any current masthead page.

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