Characterization of Met-139 as the Photolabeled Amino Acid Residue in the Steroid Binding Site of Sex Hormone Binding Globulin Using Δ^6 Derivatives of either Testosterone or Estradiol as Unsubstituted Photoaffinity Labeling Reagents†

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ABSTRACT: Immunopurified human sex hormone binding globulin (SHBG) was photoinactivated and photolabeled by radioinert and radioactive photoaffinity labeling steroids Δ^6-testosterone (Δ^6-T) and Δ^6-estradiol (Δ^6-E_2). The maximal levels of specific incorporation of these two reagents were 0.50 and 0.33 mol of label/mol of SHBG, respectively. Covalently labeled SHBG fractions were citraconylated, reduced, carboxymethylated, and cleaved by trypsin. Separation of tryptic digests by reverse-phase liquid chromatography gave single radioactive peaks at the same retention times with both steroid reagents. However, the two labeled peptidic fractions could be distinguished by capillary electrophoresis and immunodetection with anti-steroid antibodies, whereas the covalent attachment of radioactivity was confirmed by thin-layer chromatography on silica gel. Edman degradation of the two labeled peptides showed a single sequence His-Pro-Ile-([^{3}H]X)-Arg corresponding to the pentapeptide His-Pro-Ile-Met-Arg 136–140 of SHBG sequence. The coincidence, in both cases, of the absence of an identifiable amino acid residue and of the elution of the most intense peak of radioactivity at the fourth cycle of Edman degradation suggests that the same Met-139 residue was labeled by Δ^6-[1,2-$^3H_2$]T or by Δ^6-[17α-^{3}H]E_2. Liquid secondary ion mass spectrometry of the two peptides showed $[M + H]^+$ ions at m/z 939.8 or 923.8, corresponding respectively to the addition of Δ^6-T or Δ^6-E_2 to the pentapeptide. The presence of the steroid molecule in the Δ^6-[^{3}H]T–pentapeptide conjugate was confirmed by the difference of 2 mass units with the $[M + H]^+$ peak of the Δ^6-[4-^{14}C]T–pentapeptide conjugate.

The sex hormone binding globulin (SHBG),[1] also called sex steroid binding protein (SBP), is a transport glycoprotein present in blood of most mammalian species, which binds 5α-dihydrotestosterone (DHT) and testosterone (T) with high affinity and estradiol (E_2) with a weaker affinity, thus modulating the balance in the free steroid concentration, the bioavailability, and the metabolic clearance rate of the active sex steroids. More recently, it has been shown that SHBG could be recognized by specific membrane receptors of hormone target cells, in a manner depending on the interaction of sex steroids with the binding site (Rosner, 1990a; Strel'Chyonok & Avvakumov, 1990).

The structure and function of SHBG as well as the properties of two other transport proteins, androgen binding protein (ABP) and corticosteroid binding globulin (CBG), have been the object of detailed studies. The most important results of these studies have been summarized in three recent reviews (Strel'Chyonok & Avvakumov, 1990; Hammond, 1990a; Petra, 1991). Structural and binding analyses have shown that SHBG is a homodimeric glycoprotein of approximately 90 kDa, resulting from various combinations of size-heterogeneous glycosylated subunits with only one steroid binding site per mole of SHBG homodimer, thus raising the question of the spatial interaction of the asymmetric steroid ligand with two subunits having identical polypeptidic structures (Petra et al., 1986a,b). However, it has been suggested that SHBG in human serum might be present in its monomeric form (Englebienne et al., 1987). The influence of dimer dissociation and renaturation on the formation of the steroid binding site of rabbit SHBG has been studied recently by fluorescence spectroscopy, using dansyl-labeled SHBG or dihydroequilenin as fluorescent probes (Casali et al., 1990). Direct sequencing of overlapping peptide fragments of purified human SHBG (Walsh et al., 1986) or cDNA sequencing (Hammond et al., 1987; Que & Petra, 1987; Gershagen et al., 1987) established the presence of a single polypeptide, composed of 373 residues.

One characteristic feature of SHBG binding properties is the specific recognition of both androgenic and estrogenic steroids. Binding data and fluorescence investigations of the interaction of human or rabbit SHBGs with equilenin and DHT (Orstan et al., 1986) or of rabbit SHBG with dihydroequilenin, E_2, and DHT (Casali et al., 1990) strongly support the assumption that estrogens and androgens bind to the same site, despite the large structural differences existing between these ligands. The binding affinity of human SHBG for both steroids was found to decrease with increasing temperature (Shanbag & Södergard, 1986). Comparisons between species indicate that E_2 is always bound with weaker affinity than androgens with interspecies differences (Mickelson & Petra, 1978; Renoir et al., 1980) which may reflect variations in the structure of binding sites.

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[1] Abbreviations: SHBG, sex hormone binding globulin; ABP, androgen binding protein; CBG, corticosteroid binding globulin; T, testosterone; DHT, 5α-dihydrotestosterone; E_2, estradiol; Δ^6-T, 17β-hydroxyandrosta-4,6-dien-3-one; Δ^6-E_1, 3-hydroxyestra-1,3,5(10),6-tetraen-17-one; Δ^6-E_2, estra-1,3,5(10),6-tetraene-3,17β-diol; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid; DCC, dextran-coated charcoal; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography.

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Localization of the steroid binding site of human SHBG was first attempted by photoaffinity labeling with the Δ^6-[1,2-$^3H_2$]T reagent employed previously for covalent labeling of rat ABP (Taylor et al., 1980a,b), rabbit ABP (Cheng et al., 1984), human ABP (Cheng et al., 1985), rabbit SHBG (Danzo et al., 1982; Cheng et al., 1984), and human SHBG (Cheng et al., 1983a, 1985). Chromatographic separation and sequencing of labeled peptides suggested that radioactivity was concentrated in the C-terminal region beginning at Ala-296 (Hammond et al., 1987; Namkung et al., 1988; Petra et al., 1988), close to the hydrophobic region containing alternating leucines which had been proposed as a probable location of the binding site (Walsh et al., 1986; Que & Petra, 1987; Griffin et al., 1989). However, these studies were unsuccessful for identifying the covalently labeled amino acid residues. Similar experiments undertaken in our laboratory suggested that the major site of photoaffinity labeling of SHBG with the Δ^6-[^{3}H]T reagent was the Met-139 residue, a position clearly distinct from the C-terminal region (Grenot et al., 1988). This unexpected location was later supported by the results of one of two very recent studies, both using a 17β-bromoacetoxy derivative of [^{14}C]DHT as electrophilic affinity labeling reagent. While the first study (Khan & Rosner, 1990) reported the covalent labeling of His-235, the second one (Namkung et al., 1990) identified Lys-134 as the main site of specific labeling. The identification of Lys-134 and Met-139 by two different methodologies and the presence of these two residues in the vicinity of the hydrophobic peptidic segment IALGGLLFP located between Ile-141 and Pro-149 amino acid residues (Walsh et al., 1986) strongly suggest that this region could be a part of the binding site of SHBG but do not exclude contributions of other peptidic segments located either on the same monomer or on the two monomers. The location of the binding site in this hydrophobic region is also suggested by the recent isolation of the peptidic fragment IALGGLLLPTS (amino acids 171–181 of the rat ABP precursor) as the major radiolabeled peptide after photolabeling of rat ABP with the Δ^6-[^{3}H]T reagent (Danzo et al., 1991). However, in this latter case, the covalently bound radioactivity could not be assigned to individual amino acids of the isolated peptide.

The present work was undertaken in the view of characterizing unambiguously the sites of specific covalent photolabeling of human SHBG by parallel experiments with two unsubstituted androgenic and estrogenic photoaffinity labeling steroids, the known Δ^6-T reagent (Taylor, et al., 1980a) and the corresponding Δ^6-E_2 derivative, which had been previously tested without success as a photoaffinity labeling reagent of the rat uterine estrogen receptor (Katzenellenbogen et al., 1974). A preliminary report of experiments with the Δ^6-[^{3}H]T reagent has been published (Grenot et al., 1988).

EXPERIMENTAL PROCEDURES

Chemicals. [1,2,4,5,6,7-$^3H_6$]DHT (126 Ci/mmol) and [2,4,6,7,16,17-$^3H_6$]E_2 (150 Ci/mmol) were from Amersham Corp., [1,2,6,7-$^3H_4$]T (90 Ci/mmol) was from CEA (Saclay, France), and Δ^6-[1,2-$^3H_2$]T (42.4 Ci/mmol) was from NEN. DHT, T, and E_2 were from Roussel-UCLAF (Paris, France) and Δ^6-E_1 was from Sigma. Trypsin–L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK–trypsin) was from Worthington and endoproteinase Glu-C was from Boehringer (Mannheim, Germany). Trisacryl GF 05 was purchased from IBF (Villeneuve-la-Garenne, France).

Buffers. PBS was 10 mM sodium phosphate buffer, pH 7.4, and 0.15 M NaCl; PBS-gelatin was PBS containing 0.1% gelatin and 0.1% NaN_3.

Synthesis of Radioactive Photoaffinity Labeling Reagents. Δ^6-[4-^{14}C]T (~27 mCi/mmol) was prepared from [4-^{14}C]T (50 mCi/mmol, purchased from NEN) by acid-catalyzed dehydrogenation of the corresponding 17-acetate derivative with dichlorodicyanobenzoquinone (Turner & Ringold, 1967). A mixture of [4-^{14}C]T (20 μCi, ~116 μg) and radioinert T (100 μg) was acetylated by a 3:1 mixture of pyridine and acetic anhydride (100 μL) for 2 h at room temperature. The reaction product was purified by TLC on fluorescent silica gel (Merck 60 F_{254}, pentane–ethyl acetate 1:1). The purified acetylated product (180 μg, 0.55 μmol) was dissolved in anhydrous dioxane (20 μL) containing a slight excess of dichlorodicyanobenzoquinone (170 μg, 0.75 μmol). Anhydrous hydrogen chloride was then bubbled slowly (5 min) through a glass capillary into the reaction mixture at 22 °C. The reaction mixture was neutralized by addition of a saturated aqueous solution of $NaHCO_3$ (50 μL) and purified immediately, without extraction, by TLC using the solvents described above and 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). The protecting 17-acetate group was removed by saponification with 50 μL of a 1:5 mixture of 10% aqueous solution of K_2CO_3 in methanol, for 12 h at room temperature, in the dark, under a nitrogen atmosphere. The saponified product was purified by TLC on silica gel, as above, to give Δ^6-[4-^{14}C]T in about 50% overall yield. The product, purified by reverse-phase HPLC, as above, showed a radiochemical purity greater than 98%. The chromatographic properties of this radiolabeled Δ^6-[^{14}C]T were identical to those of commercial Δ^6-T, in both TLC and HPLC separation systems, thus confirming the structure of the labeled product. The labeled Δ^6-[^{14}C]T could be stored in the dark at –20 °C, in absolute ethanol for 3 months without significant degradation.

The Δ^6-[17α-^{3}H]E_2 derivative (~4.4 Ci/mmol) was prepared by reduction of an ice-cooled solution of Δ^6-E_1 (300 μg, 1.12 μmol in 100 μL of ethanol) with a solution of [^{3}H]$NaBH_4$ in 0.1 M sodium hydroxide (50 μL, 5 mCi, specific activity 17.5 Ci/mmol, purchased from Amersham), under argon atmosphere. After 1.5 h, the reaction was stopped by addition of acetone (300 μL). One hour later, the reaction mixture was acidified at pH 4.5 with 1 M acetic acid and the steroids were extracted into ethyl acetate. The reaction product was purified by TLC on silica gel (Merck 60 F_{254}, toluene–ethyl acetate 1:3) and could be stored in the dark, at –20 °C, in absolute ethanol, for 3 months without significant degradation. This fraction was purified 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) and showed a radiochemical purity greater than 95%. The specific activity of Δ^6-[17α-^{3}H]E_2 (~4.4 Ci/mmol) was estimated as one-fourth that of the commercial [^{3}H]$NaBH_4$. The structure of Δ^6-[17α-^{3}H]E_2 was confirmed by the identity of its chromatographic properties with those of unlabeled Δ^6-E_2, which was also prepared by the same method. Unlabeled Δ^6-E_2 was characterized by its UV absorbance in 95% ethanol [λ_{max} at 304 nm (ϵ = 2400 M^{-1} cm^{-1}), 270 nm (ϵ = 5300 M^{-1} cm^{-1}), 262 nm (ϵ = 5900 M^{-1} cm^{-1})] and by nuclear magnetic resonance [^{1}H-NMR (C_5D_5N) δ 0.98 (3 H, s, CH_3-18), 3.95 (1 H, t, J = 8 Hz, H-17), 6.04 and 6.64 (2 H, 2d, J = 10 Hz, H-6 and H-7), 7.13–7.30 (3 H, m, aromatic H)], which were in good agreement with the corresponding spectral characteristics of commercial Δ^6-E_1.

Purification of SHBG. Human SHBG was isolated from an aqueous solution of an alcoholic precipitate of placental blood pools (provided by Pasteur-Merieux, Marcy-l'Etoile,

France). The concentration of SHBG in this fraction (45 nM) was estimated by an immunoradiometric assay (^{125}I-SBP CoatRIA kit, bioMérieux, Marcy-l'Etoile, France) and confirmed by a DHT binding capacity assay (Hammond et al., 1985). SHBG was purified from this placental blood fraction by immunopurification using a monoclonal antibody obtained in our laboratory against human SHBG (Grenot and Cuilleron, unpublished results) and immobilized on Sepharose 4B. The placental blood fraction (3 L) was incubated batchwise with the affinity resin (100 mL) for 2 h at 4 °C with mild agitation. After filtration of the unretained fraction, the gel was washed several times, in batch, at 4 °C, alternatively with 500 mL of two buffers [20 mM Tris-HCl, pH 7.4, 1 M KCl, and 10% dimethylformamide (Cheng et al., 1983a) or 10 mM sodium phosphate, pH 7.4, 0.15 M NaCl, and 0.2% Tween 20] until the protein content could not be detected by UV absorbance at 220 nm. The affinity resin was then equilibrated with 500 mL of 50 mM NH_4HCO_3, pH 7.5, and SHBG was eluted by 450 mL of 1 M NH_4OH. After addition of 0.5% glycerol, the NH_4OH eluate was immediately frozen and lyophilized. SHBG eluted from affinity resin was further purified by anion-exchange HPLC on a DEAE-5PW column (0.75 × 7.5 cm, Waters Associates) using a linear gradient of 1 M NaCl in 10 mM sodium phosphate buffer, pH 7.5 (0–30% in 30 min). These two purification steps gave an overall yield of 60–70% determined with the immunoradiometric assay kit and confirmed by the DHT binding assay. The purified SHBG fraction collected from the DEAE column was concentrated rapidly under vacuum in a Speed-Vac concentrator and transferred in PBS by exclusion chromatography on Trisacryl GF 05. Purified SHBG was then stored at 4 °C, in PBS containing 10% glycerol, at a concentration of 2 mg/mL (21.4 μM), measured by UV absorption at 280 nm using the reported values of ϵ = 114 000 M^{-1} cm^{-1} and MW = 93 400 (Petra et al., 1986b).

Binding Measurements. The binding capacity of unpurified SHBG was measured by a dextran-coated charcoal (DCC) saturation assay with [^{3}H]DHT in which the nonspecific binding was estimated in the presence of an excess of radioinert DHT and cortisol (Hammond et al., 1985). The binding capacity of immunopurified SHBG was measured on aliquots (10 μL) of a 1/10 000 dilution of the initial SHBG solution (2 mg/mL) in PBS-gelatin, as above, but in the absence of added cortisol.

The association constants of SHBG with DHT, T, E_2, Δ^6-T, and Δ^6-E_2 were determined by the method of Scatchard (1949) using a separation by DCC (unpurified and purified SHBGs) or by equilibrium dialysis (purified SHBG only). (1) Separation by DCC: The bound concentrations were determined after incubation of aliquots (100 μL) of a solution of purified SHBG (5 nM) in PBS-gelatin buffer or of similar concentrations of unpurified SHBG with eight concentrations (ranging from 0.2 to 10 nM) of each of the corresponding ^{3}H-labeled steroids (specific activities were all adjusted to ~4.4 Ci/mmol with unlabeled steroid) in 100 μL of PBS-gelatin, for 1 h at 22 °C and 15 min at 4 °C, followed by treatment with 0.5 mL of DCC at 4 °C and centrifugation (3500 rpm for 15 min at 4 °C). (2) Equilibrium dialysis: Aliquots (1 mL) of immunopurified SHBG (9 nM) in PBS-gelatin buffer were placed inside dialysis bags and dialyzed for 20 h at 25 °C against eight concentrations (ranging from 2 to 40 nM) of ^{3}H-labeled steroids (specific activities as above), placed outside the bag in 10 mL of PBS buffer.

Photoaffinity Labeling. The conditions of photoaffinity labeling were essentially those previously described (Taylor et al., 1980a,b; Petra et al., 1988; Namkung et al., 1988; Grenot et al., 1988). Immunopurified SHBG was first incubated for 1 h at 22 °C and 15 min at 4 °C, under argon atmosphere, in the dark, with the different Δ^6-steroid photolabeling reagents and then transferred in NMR glass tubes (0.4 × 18 cm) placed at a distance of 3 cm from the external wall of an efficiently refrigerated high-pressure mercury lamp (Hanovia, 450 W) and maintained at 4 °C. The solutions were first degassed by bubbling a weak stream of argon through a glass capillary and then irradiated under argon atmosphere at $\lambda > 300$ nm, using a 2-mm-thick Pyrex filter (Taylor et al., 1980a), or at $\lambda > 315$ nm, using a 30% $CuSO_4$ liquid filter (Payne et al., 1980). After irradiation, the dissociation of noncovalently bound steroids 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, and 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, Δ^6-E_2, Δ^6-T acetate, Δ^6-progesterone, or DHT (1 nmol) in 100 μL of PBS-gelatin buffer. (2) Kinetics of covalent photoattachment: SHBG (0.1 nmol) was incubated with Δ^6-T (0.1 nmol) containing Δ^6-[^{3}H]T (10^4 cpm) or Δ^6-E_2 (0.2 nmol) containing Δ^6-[^{3}H]E_2 (10^4 cpm), in 100 μL of PBS-gelatin buffer, in the absence or in the presence of DHT (10 or 20 nmol, respectively). (3) Determination of HPLC profiles of tryptic or endoproteinase Glu-C digests: (a) SHBG (2 nmol) was incubated with Δ^6-T (2 nmol) containing Δ^6-[^{3}H]T (10^5 cpm) or with Δ^6-E_2 (4 nmol) containing Δ^6-[^{3}H]E_2 (10^5 cpm), in 200 μL of PBS (without gelatin) in the absence or in the presence of DHT (200 or 400 nmol, respectively); (b) SHBG (2 nmol) was incubated with Δ^6-[^{14}C]T acetate (2 nmol); (c) heat-denatured SHBG (2 nmol) was incubated with Δ^6-T (2 nmol) containing Δ^6-[^{3}H]T (10^5 cpm). (4) Determination of evolution with time of the radioactivity profiles of tryptic digests: SHBG (2 nmol) was incubated with Δ^6-T (0.4 nmol) containing Δ^6-[^{3}H]T (10^5 cpm) or with Δ^6-E_2 (0.4 nmol) containing Δ^6-[^{3}H]E_2 (10^5 cpm), in 200 μL of PBS (without gelatin). (5) Sequence analysis: (a) SHBG (10 nmol) was incubated with Δ^6-T (10 nmol) containing Δ^6-[1,2-$^3H_2$]T (8 × 10^4 cpm, 2.1 pmol) in 1 mL of PBS; (b) SHBG photolabeled with Δ^6-[^{14}C]T was prepared as described below for mass determination; (c) SHBG (20 nmol) was incubated with Δ^6-E_2 (40 nmol) containing Δ^6-[17α-^{3}H]E_2 (3.2 × 10^5 cpm, 83 pmol) in 1.5 mL of PBS. (6) Molecular mass determinations: (a) SHBG (50 nmol) was incubated with Δ^6-T (50 nmol) containing Δ^6-[1,2-$^3H_2$]T (10^6 cpm) in 2.5 mL of PBS; (b) SHBG (50 nmol) was incubated with Δ^6-[4-^{14}C]T (50 nmol of the above described tracer) in 2.5 mL of PBS; (c) SHBG (150 nmol) was incubated with Δ^6-E_2 (300 nmol) containing Δ^6-[17α-^{3}H]E_2 (10^6 cpm) in 7.5 mL of PBS.

Gel Electrophoresis. Native polyacrylamide gel electrophoresis (Davis, 1964) and sodium dodecyl sulfate (NaDodSO$_4$)–polyacrylamide gel electrophoresis (Laemmli, 1970) were performed in a vertical slab-gel apparatus. Unlabeled SHBG was stained with silver nitrate (Heukeshoven & Dernick, 1985), whereas photoaffinity-labeled SHBG was detected by fluorography, after treatment of the gel with 1 M sodium salicylate, using a Kodak X-OMAT film. Molecular weight markers (myosin, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme) were purchased from Amersham.

Enzymatic Hydrolysis of Labeled SHBG and Purification of Peptidic Fragments. Photolabeled SHBG was esterified with citraconic anhydride in guanidinium chloride buffer (Glazer et al., 1975). Citraconylated samples were reduced with dithioerythritol, carboxymethylated by iodoacetic acid, and transferred in 0.5 M Tris-HCl buffer, pH 8.2, by exclusion chromatography. Cleavage with TPCK–trypsin (1% enzyme/SHBG ratio, w/w) was accomplished by incubation for 24 h at 37 °C (Grenot et al., 1988). 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 successive HPLC purifications in the presence of 0.1% TFA in the elution gradient, for the first and third separation, and in the presence of 20 mM NH_4HCO_3, pH 7.5, in the aqueous fraction of the gradient for the second separation.

SHBG photolabeled with Δ^6-[^{3}H]T was also cleaved by trypsin without prior citraconylation (Grenot et al., 1988). In this case, the HPLC separation of the tryptic digest in the acidic conditions described above showed the presence of a major radioinert peptide contaminant which was purified by two further HPLC separations under the same conditions.

The cleavage of SHBG photolabeled with Δ^6-[^{3}H]T was also performed, without a prior citraconylation step, by incubation with endoproteinase Glu-C (5% enzyme/SHBG ratio, w/w) for 6 h at 37 °C, in 0.1 M sodium phosphate buffer, pH 7.8. The cleavage products were purified by HPLC, as described above for the radioinert peptide contaminant.

Binding of Photoaffinity-Labeled Peptides to Anti-Steroid Antibodies. Aliquots (10 000 cpm) of purified Δ^6-[^{3}H]T and Δ^6-[^{3}H]E_2 peptides were incubated with 100 μL of a 1/100 dilution of anti-7-*O*-(carboxymethyl)oximino-DHT antibodies (Grenot & Cuilleron, 1979) and anti-6-*O*-(carboxymethyl)-oximino-E_2 antibodies (Grenot & Cuilleron, unpublished results) in 0.5 mL of PBS-gelatin for 2 h at 37 °C and 15 min at 4 °C, in the presence and in the absence of an excess of DHT or E_2 (0.1 μg). Radioactivity bound to antibodies was measured after addition of 1 mL of DCC suspension and centrifugation.

Capillary Electrophoresis. Capillary electrophoresis of purified Δ^6-[^{3}H]T and Δ^6-[^{3}H]E_2 peptides was performed on uncoated capillary glass tubes with an automatic apparatus (Applied Biosystems 270 A). Two buffers were tested: 20 mM sodium citrate, pH 2.5, and 10 mM sodium phosphate, pH 6.0, and 20 mM $NaDodSO_4$. UV peaks were recorded at 214 nm and retention times were measured by comparison with that of an internal standard of dimethyl sulfoxide.

Sequence Determinations. Edman sequencing of purified peptides was performed with a gas-phase sequencer equipped with on-line phenylthiohydantoin (PTH) analyzer (Applied Biosystems, Model 470 sequencer).

Amino Acid Analysis. Amino acid composition was determined after acid hydrolysis of the purified peptide (1 nmol) by 6 N HCl, in an evacuated sealed tube, at 110 °C for 24 h, in the presence of 1% phenol. The hydrolysate was separated on a reverse-phase HPLC C_{18} column (Merck, LiChrospher 100 RP-18, 5 μm), at 50 °C, after phenyl isothiocyanate derivatization of amino acids, using a multistep gradient from 0 to 100% solvent B (solvent A was 25 mM sodium acetate and 0.1% triethylamine, pH 6.0; solvent B was 50% CH_3CN in solvent A).

Mass Spectrometry. Liquid secondary ion mass spectrometry experiments were performed on a VG ZAB SEQ mass spectrometer equipped with a Cs^+ gun operating at 40 keV. The accelerating voltage was 8 kV and the instrumental resolution was 2000 (10% valley). Calibration was performed with $(CsI)_nCs^+$ cluster ions. Measurements were performed using a stainless steel probe tip by adding approximately 1 nmol of peptide sample dissolved in 2 μL of 5% aqueous acetic acid to 2 μL of matrix (2:1 mixture of glycerol/1-thioglycerol acidified with 1% TFA). Single-scan spectra were recorded in the mass range 100–2000.

RESULTS

Purification and Characterization of SHBG. Steroid-free human SHBG was isolated in 60–70% yield from an alcoholic precipitate of placental blood pools by immunopurification with an immobilized monoclonal anti-human SHBG antibody, followed by anion-exchange chromatography. All attempts to augment the number of binding sites of unpurified SHBG by stripping experiments with activated charcoal were unsuccessful, thus indicating the absence of significant amounts of residual endogenous steroids bound to SHBG in the initial placental blood fraction.

The number of steroid binding sites of immunopurified SHBG for DHT, estimated either by a dextran-coated charcoal (DCC) saturation assay (Hammond et al., 1985) (18.5 nmol/mL) or from the abscissa intercept of Scatchard plots (Scatchard, 1949) established after equilibrium dialysis experiments (18.7 nmol/mL), were in good agreement with the corresponding SHBG concentration as measured by a commercial immunoradiometric assay (20.0 nmol/mL) or as estimated from UV absorption at 280 nm (21.4 nmol/mL). No significant loss of binding activity of immunopurified SHBG could be observed up to 2 months of continuous storage at 4 °C. However, longer periods of storage may result in a diminution of the binding capacity of purified SHBG (Cheng et al., 1983b). The association constant for DHT binding to immunopurified SHBG at 22 °C (2.2×10^9 M^{-1}), estimated from Scatchard plots established after DCC separation, was higher than the association constants measured in parallel for T (0.9×10^9 M^{-1}) or E_2 (0.5×10^9 M^{-1}). These values were corroborated by the measurement in similar conditions of the equilibrium constants for the binding of these three steroids to SHBG in a pool of human sera (DHT, 2.2×10^9 M^{-1}; T, 0.9×10^9 M^{-1}; E_2, 0.5×10^9 M^{-1}). These values were also found to agree in magnitude with the equilibrium constants previously reported for the binding of DHT with unpurified human SHBG (Mickelson & Petra, 1974; Renoir et al., 1980) and for the binding of the three former DHT, T, or E_2 steroid ligands with unpurified SHBG from human plasma (Shanbag & Södergard, 1986). These binding measurements suggest that the immunopurification protocol had no significant effects on the steroid-binding properties of SHBG. The association constants of immunopurified SHBG measured at 25 °C by equilibrium dialysis were significantly higher for DHT (3.7×10^9 M^{-1}) and T (1.6×10^9 M^{-1}), while a lesser increase was observed for E_2 (0.6×10^9 M^{-1}).

Immunopurified SHBG was also characterized by Edman sequencing of three different peptidic fragments. Sequence analysis of the N-terminal part of immunopurified SHBG (32 amino acid residues), of one tryptic fragment (17 amino acid residues from Met-107 to Arg-123) isolated in the absence of citraconylation of SHBG, and of the N-terminal fragment (24 amino acid residues from Val-121 to Gly-144) of the peptide obtained after cleavage with endoproteinase Glu-C showed no difference with the corresponding sequences established for SHBG purified from human blood plasma (Walsh et al., 1986).

Characterization of the Binding of Δ^6-T and Δ^6-E_2 to Immunopurified SHBG. The association constants estimated

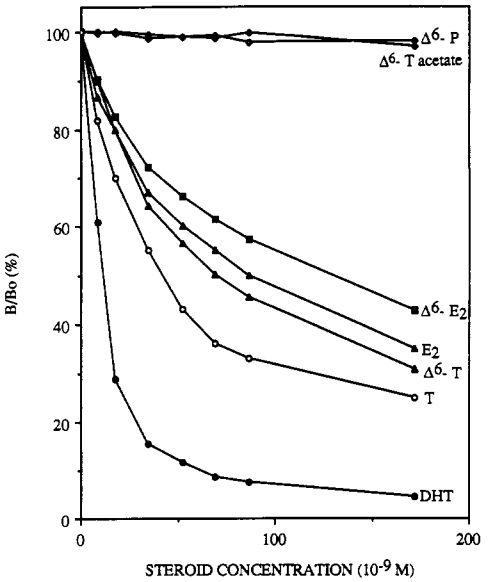

FIGURE 1: Competitive binding assays for DHT and Δ^6-steroid photoaffinity labeling reagents with immunopurified SHBG. Aliquots (100 μL) of purified SHBG (2.0 nM in PBS-gelatin) were incubated with [^{3}H]DHT (50 000 cpm, 4.5×10^{-4} nmol) and seven concentrations (ranging from 10 to 180 nM) of DHT and of each of the radioinert T, E_2, Δ^6-T, Δ^6-E_2, Δ^6-T acetate, and Δ^6-progesterone competitors in 400 μL of PBS-gelatin for 1 h at 22 °C and 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 (○), E_2 (△), Δ^6-T (▲), Δ^6-E_2 (■), Δ^6-T acetate (◆), Δ^6-progesterone (◇).

for Δ^6-T binding to SHBG (0.35×10^9 M^{-1} at 22 °C after DCC separation and 1.1×10^9 M^{-1} at 25 °C after equilibrium dialysis) and the corresponding values for Δ^6-E_2 (0.2×10^9 and 0.4×10^9 M^{-1}) were lower than those estimated above for T and E_2 analogs. The abscissa intercepts of the linear Scatchard plots of these two ligands and of those of T, DHT, and E_2 were the same within experimental error, thus suggesting the binding of all these derivatives at the same site. However, the number of binding sites estimated from measurements with the DCC separation method was about half that obtained by equilibrium dialysis, owing probably to rapid dissociation in the presence of charcoal.

Displacement experiments of [^{3}H]DHT bound to SHBG (Figure 1) showed that the presence of a Δ^6 double bond led to a lower cross-reactivity with SHBG when compared with that of the corresponding T and E_2 analogs, whereas no significant competitive effects were observed with Δ^6-T acetate and Δ^6-progesterone in the concentration range studied (up to 200-fold molar excess). Conversely, displacement of the binding activity of SHBG for each of the [^{3}H]T, Δ^6-[^{3}H]T, and Δ^6-[^{3}H]E_2 tracers by a 200-fold molar excess of DHT or of the three corresponding nonradioactive analogs led to a decrease of more than 90% of bound radioactivity (data not shown).

The $\Delta^{9(11)}$-E_2 derivative was also tested in parallel to Δ^6-E_2 as another potential photolabeling reagent of SHBG, but it failed to compete with [^{3}H]DHT for binding to SHBG (data not shown).

Time Course of Photolysis of Δ^6-T and Δ^6-E_2 Photoreagents. Photolysis of Δ^6-T and of Δ^6-E_2 at $\lambda > 300$ nm, using a 2-mm-thick Pyrex filter (Taylor et al., 1986a), followed by recording of the UV absorption spectra of the photolyzed samples as a function of irradiation time, was complete by about 30 min (Figure 2). Similar results (data not shown)

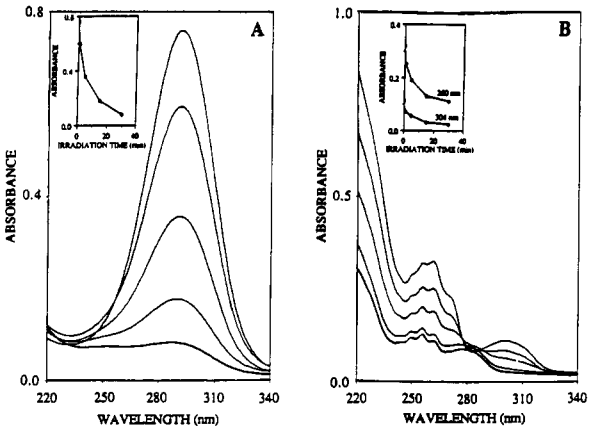

FIGURE 2: Time course of photolysis of Δ^6-T and Δ^6-E_2. Aliquots (1 mL) of aqueous solutions (PBS containing 0.5% ethanol) of Δ^6-T and Δ^6-E_2 (10 μg/mL) were irradiated at 4 °C, under argon atmosphere, in the apparatus described in Experimental Procedures. Absorbance spectra were recorded between 220 and 340 nm immediately after irradiation, following exposure at $\lambda > 300$ nm for 0, 1, 5, 15, and 30 min. (A) Δ^6-T. (B) Δ^6-E_2.

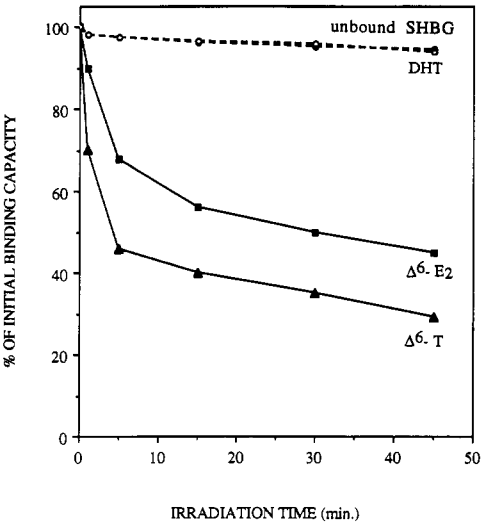

FIGURE 3: Photoinactivation of immunopurified SHBG by Δ^6-T and Δ^6-E_2. Irradiation of SHBG incubated with Δ^6-T (▲), Δ^6-E_2 (■), or DHT (●) (as described in Experimental Procedures) and irradiation of unbound SHBG (○) were performed at $\lambda > 300$ nm for 0, 1, 5, 15, 30, and 45 min. Noncovalently bound steroids were removed by exclusion chromatography (see Experimental Procedures). The SHBG binding activity was restored by chromatography of the protein-containing fractions on microcolumns (Trisacryl GF 05) equilibrated with PBS-gelatin buffer and measured directly on aliquots of the protein-containing eluate (50 μL) incubated with [^{3}H]DHT (50 000 cpm) for 1 h at 22 °C. Free and bound steroids were then separated by DCC.

were obtained by irradiation at $\lambda > 315$ nm, using a 30% $CuSO_4$ liquid filter (Payne et al., 1980).

Kinetics of Photoinactivation. After 30 min of irradiation at $\lambda > 300$ nm, about 65% and 50% of initial binding capacity of SHBG for [^{3}H]DHT was inactivated using a 10:1 steroid/protein molar ratio of the radioinert Δ^6-T and Δ^6-E_2 reagents, respectively (Figure 3). Photoinactivation experiments with Δ^6-T acetate and Δ^6-progesterone, which both have a much lower affinity for SHBG than Δ^6-T, were found to exert no discernible inhibiting effects on the subsequent binding of [^{3}H]DHT (data not shown).

The much slower photoinactivation process observed, in both cases, after 15–30 min, corresponded probably, for a large part, to direct photodenaturation of SHBG binding sites, which culminated at 5–10% of the initial binding capacity,

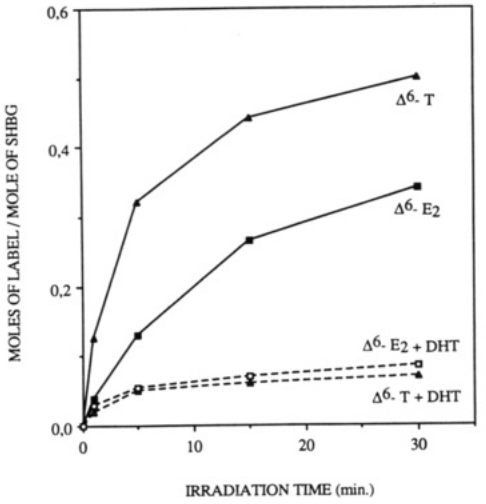

FIGURE 4: Kinetics of covalent photoattachment of Δ^6-[^{3}H]T and Δ^6-[^{3}H]E_2 to immunopurified SHBG. Irradiation of SHBG incubated with Δ^6-[^{3}H]T (▲) or Δ^6-[^{3}H]E_2 (■) in the absence of DHT or in the presence (open symbols) of radioinert DHT (as described in Experimental Procedures) was performed at $\lambda > 300$ nm for 1, 5, 15, and 30 min. For each irradiation time, the stoichiometry of labeling is expressed as moles of label incorporated per mole of SHBG (dimer). The two upper curves (solid lines) were obtained after subtraction of nonspecific labeling (dotted lines).

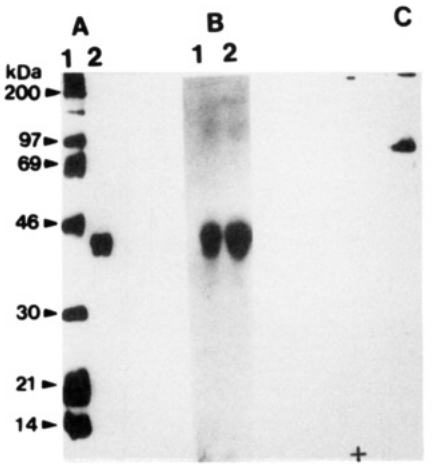

FIGURE 5: Polyacrylamide gel electrophoresis of photolabeled and unlabeled samples of immunopurified SHBG. $NaDodSO_4$–polyacrylamide (10% acrylamide) gel electrophoresis: (A) Molecular weight markers (lane 1) and unlabeled SHBG (lane 2) stained with $AgNO_3$; (B) SHBG photolabeled with Δ^6-[^{3}H]T (lane 1) or Δ^6-[^{3}H]E_2 (lane 2) and revealed by fluorography. Native polyacrylamide (7.5% acrylamide, 2.5% cross-linking) gel electrophoresis: (C) Immunopurified SHBG stained with $AgNO_3$.

after 45 min of irradiation, for both unbound and DHT-bound SHBGs. The stability of SHBG to irradiation at $\lambda > 300$ nm is in good agreement with that reported for rat ABP (Taylor et al., 1980a) in similar photoinactivation conditions. This stability contrasted with the rapid loss of estrogen binding capacity observed for α-fetoprotein at $\lambda > 300$ nm but not at $\lambda > 315$ nm (Payne et al., 1980). The stability of unbound SHBG to photolysis at $\lambda > 300$ nm validates photolabeling protocols using an excess of protein, since the excess of steroid-free binding sites is not immediately destroyed by direct photolysis.

Kinetics of Covalent Photoattachment of Δ^6-[^{3}H]T and Δ^6-[^{3}H]E_2. The time course of photoaffinity labeling was studied at $\lambda > 300$ nm, using a 1:1 steroid/protein molar ratio with Δ^6-[^{3}H]T or a 2:1 steroid/protein molar ratio with Δ^6-[^{3}H]E_2 (Figure 4). The maximal levels of both total and specific (i.e., inhibited by an excess of DHT competitor) covalently attached radioactivity were reached after 30 min of irradiation in both cases. The highest levels of specific attachment of Δ^6-[^{3}H]T and Δ^6-[^{3}H]E_2 corresponded respectively to 0.50 and 0.33 mol of label/mol of SHBG, whereas only low and nonspecific labeling occurred in similar conditions with tritiated T, DHT, and E_2 ligands. Control experiments without irradiation showed a total absence of irreversibly bound radioactivity, thus indicating that photoactivation is necessary for covalent labeling. Increasing the relative amount of steroid from a 1:1 (Δ^6-[^{3}H]T) or 2:1 (Δ^6-[^{3}H]E_2) ratio up to a 10:1 ratio of steroid/protein concentrations did not augment significantly the amounts of specific labeling but increased the difficulties for preparative chromatographic separation of covalently bound radioactivity, whereas irradiations at $\lambda > 315$ nm led to lower yields in the time range studied, especially in the case of the Δ^6-[^{3}H]E_2 photoreagent (data not shown).

Gel Electrophoresis of Photolabeled SHBG. The radioactivity distribution after $NaDodSO_4$–polyacrylamide gel electrophoresis of SHBG samples photolabeled with Δ^6-[^{3}H]T and Δ^6-[^{3}H]E_2 ligands was found exclusively at the level of the characteristic bands of pure unlabeled SHBG (Figure 5). The absence of any significant effects on the electrophoretic mobility of SHBG monomers indicates that the photoaffinity labeling with the two steroid reagents induced no modification of the charge and produced no protein cleavage. A native polyacrylamide gel electrophoresis of pure unlabeled SHBG is also shown to confirm that immunopurified SHBG employed for photolabeling experiments was present exclusively in its dimeric form.

Enzymatic Hydrolysis of Photolabeled SHBG. The covalently labeled fractions of SHBG were citraconylated to prevent cleavage by trypsin at lysine residues, 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 profiles of samples photolabeled with radioactive Δ^6-T or Δ^6-E_2 showed in both cases a major single peak at similar retention times which was no longer detectable when a 100-fold molar excess of DHT was added to the Δ^6 photolabels. Two other control experiments were made on tryptic fragments of SHBG irradiated in the presence of the Δ^6-[^{14}C]T acetate reagent alone (1:1 steroid/protein molar ratio), which has a very low affinity for SHBG, and on tryptic fragments of heat-denatured SHBG (1 h at 60 °C) irradiated in the presence of the high-affinity Δ^6-[^{3}H]T reagent. In these control experiments, no radioactive peak could be found at the expected HPLC retention times.

The specific character of the photoattachment of the two Δ^6-[^{3}H]T and Δ^6-[^{3}H]E_2 reagents to SHBG was also established by following the evolution with the irradiation time of the distribution of the specific covalently bound radioactivity in HPLC profiles of primary separation of tryptic digests. These experiments were undertaken with a large excess of SHBG (1:5 steroid/protein molar ratio) in order to minimize nonspecific binding. The HPLC radioactivity profiles obtained after photoaffinity labeling with either Δ^6-[^{3}H]T or Δ^6-[^{3}H]-E_2 showed that, in the 1–30-min irradiation time range studied, the radioactivity was always concentrated in the same single peaks as those characterized above. When the Δ^6-[^{3}H]T reagent was employed, the intensity of the radioactive peak progressed with the irradiation time up to 100% incorporation of the added radioactivity after 30 min, whereas in similar conditions, the Δ^6-[^{3}H]E_2 photolabel led to a maximum value corresponding to only 70% of the added radioactivity.

Table I: Effects of Inert Atmosphere and of Scavengers on the Intensity of Radioactive Tryptic Peptides Separated by Reverse-Phase HPLC

incubation conditions[a]	radioactivity in tryptic peptides[b] (cpm)	
	Δ^6-[^{3}H]T-peptide	Δ^6-[^{3}H]E_2-peptide
PBS + argon stream	12500	11800
PBS + N_2 stream	3850	3354
PBS + air stream	480	395
50 mM Tris-HCl, pH 7.4[c]	11950	11000
PBS + 4-aminobenzoate (15 mM)[c]	12340	12040
PBS + glutathione (15 mM)[c]	13150	11750
PBS + 2-mercaptoethanol (15 mM)[c]	11220	10850

[a] In all cases, SHBG (2 nmol) was incubated with radioinert Δ^6-T (0.4 nmol) or Δ^6-E_2 (0.4 nmol) containing the corresponding radioactive tritiated tracers (2×10^4 cpm) in 200 μL of buffer and then irradiated for 30 min. [b] Tryptic digests were separated on a C_{18} column (Nucleosil 5 μm, 0.46 × 15 cm) and eluted at 1 mL/min using a linear aqueous acetonitrile gradient in the presence of 0.1% TFA (0–70% CH_3CN in 60 min). Fractions were collected at 1-min intervals and assayed for radioactivity. [c] Experiments performed under argon atmosphere.

Another enzyme, endoproteinase Glu-C, was also tested to obtain longer radioactive peptides from SHBG. This enzyme was employed only in the case of SHBG photolabeled with Δ^6-[^{3}H]T, after exclusion chromatography, reduction, and carboxymethylation of the labeled protein. The HPLC profile of the cleavage products showed that the radioactivity was also concentrated in one major peak.

Effects of Inert Atmosphere and of Scavengers on Photolabeling Efficiency. The effects of argon, nitrogen, or air atmosphere and those of scavengers were studied by comparing the amounts of radioactivity incorporated in the specifically radiolabeled peak obtained after HPLC fractionation of tryptic digests of immunopurified SHBG photolabeled with Δ^6-[^{3}H]T and Δ^6-[^{3}H]E_2 after incubation with a 1:5 steroid/protein molar ratio (Table I). The use of an argon atmosphere was found to augment significantly the amount of specific covalent labeling with the two Δ^6-T and Δ^6-E_2 reagents as compared with reactions performed previously under a nitrogen atmosphere (Grenot et al., 1988), owing probably to traces of oxygen in the commercial nitrogen employed in early experiments, as suggested by the almost total inhibition of specific covalent photolabeling when air was bubbled through the reaction mixture. The use, in photoaffinity labeling experiments, of a 50 mM Tris-HCl buffer, pH 7.4, or of PBS in the presence of a 15 mM concentration of other scavenger molecules such as 4-aminobenzoate, glutathione, and 2-mercaptoethanol (Bayley, 1983), showed no modifications as compared with photoaffinity labeling experiments in PBS alone.

Purification of Photoaffinity-Labeled Peptides. The separation of the pure radioactive peptides from tryptic digests of SHBG photolabeled with Δ^6-[^{3}H]T or with Δ^6-[^{3}H]E_2 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 (Figure 6). This protocol of separation confirmed the presence of a single radioactive peptide peak at similar retention times for the two tryptic digests. The purification yields are illustrated by preparative separations of radioactive tryptic peptides obtained from 50 nmol of SHBG irradiated either with Δ^6-[^{3}H]T (1:1 steroid/protein molar ratio) or with Δ^6-[^{3}H]E_2 (2:1 steroid/protein molar ratio), which led respectively to approximately 10 and

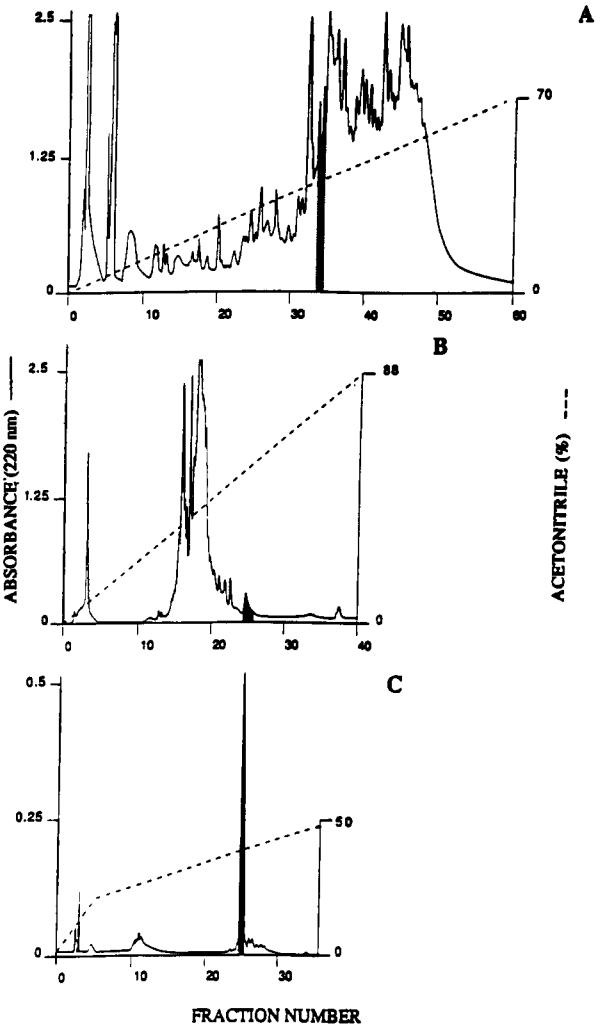

FIGURE 6: Reverse-phase HPLC purification of radioactive peptides from tryptic digests of SHBG photolabeled with Δ^6-[^{3}H]T and Δ^6-[^{3}H]E_2. (A) The tryptic digests were applied (<75 nmol of SHBG/injection) on a C_{18} column (Nucleosil 10 μm, 0.72 × 25 cm) and eluted at 2 mL/min using a linear aqueous acetonitrile gradient in the presence of 0.1% TFA (0–70% CH_3CN in 60 min). Peptide elution was monitored at 220 nm. The radioactivity was measured on aliquots (5 μL) of each 2-mL fraction. (B) Radioactive fractions from the first HPLC purification were pooled and concentrated under vacuum. The pool was applied on a C_{18} column (Nucleosil 5 μm, 0.45 × 15 cm) and eluted at 1 mL/min using a linear 20 mM aqueous NH_4HCO_3, pH 7.5–acetonitrile gradient (0–100% CH_3CN in 45 min). The radioactivity was measured on aliquots (5 μL) of each 1-mL fraction. (C) Radioactive fractions from the second HPLC purification were pooled and concentrated under vacuum. The pool was applied on a C_{18} column (Nucleosil 5 μm, 0.46 × 15 cm) and eluted at 1 mL/min using two successive linear aqueous acetonitrile gradients in the presence of 0.1% TFA (0–20% CH_3CN in 5 min and 20–45% CH_3CN in 30 min). The radioactivity was measured on aliquots (2 μL) of each 0.1-mL fraction. The shaded areas represent the radioactive peptides.

5 nmol of pure steroid–peptide conjugates. All attempts to distinguish these two radioactive tryptic peptides by HPLC failed, despite the structural difference between the two Δ^6-[^{3}H]T and Δ^6-[^{3}H]E_2 ligands.

Therefore, attempts were made to determine whether the two corresponding Δ^6-[^{3}H]T and Δ^6-[^{3}H]E_2 steroid–peptide conjugates could be distinguished by capillary electrophoresis. The use of a 10 mM sodium phosphate buffer, pH 6.0, containing 20 mM $NaDodSO_4$ led to a significant difference between the retention time of Δ^6-T–peptide conjugate (27 min) and that of Δ^6-E_2–peptide conjugate (30 min), whereas the use of 20 mM sodium citrate buffer, pH 2.5, failed to

Table II: Amino Acid Sequence Analysis of Photolabeled Tryptic Peptides

		Δ^6-[^{3}H]T-peptide, 25 000 dpm (1082 pmol)[a]				Δ^6-[^{14}C]T-peptide, 35 000 dpm (594 pmol)[a]				Δ^6-[^{3}H]E_2-peptide, 40 500 dpm (876 pmol)[a]			
			radioactivity (dpm)				radioactivity (dpm)				radioactivity (dpm)		
cycle	amino acid	PTH deriv (pmol)	S_1, S_2 wash[b]	PTH elution	S_3 wash[b]	PTH deriv (pmol)	S_1, S_2 wash	PTH elution	S_3 wash	PTH deriv (pmol)	S_1, S_2 wash	PTH elution	S_3 wash
B[c]			1806	<30	1185		2108	<30	1204		1662	<30	448
1	H	337 (+22[d])	4118	1409	188	111 (+20[d])	4171	1806	529	296 (+43[d])	5340	454	1158
2	P	355 (+100[d])	2967	1138	168	231 (+34[d])	2748	1625	334	318 (+126[d])	3874	1200	1070
3	I	329 (+121[d])	1697	740	1844	177 (+35[d])	1483	1033	2756	284 (+116[d])	1838	862	2910
4		196[e]	644	4532	276	146[e]	1547	8616	478	182[e]	796	8426	497
5	R	43 (+17[d])	149	1519	121	23 (+16[d])	200	2589	183	45 (+19[d])	185	1924	174
6			69	764	101		90	900	132		47	333	95
7			42	132	60		50	394	109		<30	120	43

[a] Picomoles of labeled peptide applied to the sequencer, calculated from the specific activity of the corresponding radioactive photoreagent. [b] Washing steps performed with *n*-heptane (S_1) and ethyl acetate (S_2), after derivatization with phenyl isothiocyanate, and with 1-chlorobutane (S_3), after elution of the PTH derivative. [c] Blank Edman cycle (B) performed without TFA cleavage and with the S_3 wash before S_1, S_2 washes. [d] Picomoles of additional PTH derivative measured at the next Edman cycle. [e] Picomoles of amino acid estimated from the radioactivity eluted at the fourth Edman cycle.

separate the two compounds. The two conjugates appeared as single peaks, at two different retention times, thus showing that two distinct homogeneous conjugates were obtained.

The covalent attachment of radioactivity of these two Δ^6-[^{3}H]T and Δ^6-[^{3}H]E_2 steroid–peptide conjugates was confirmed by TLC on silica gel, using different systems of solvent mixtures. The two radioactive peptides were observed to migrate both at the same R_f of 0.2, using the polar chloroform/methanol/NH_4OH 6:2:1 mixture. On the other hand, the use of petroleum ether/ethyl acetate, toluene/ethyl acetate, or chloroform/methanol mixtures usually employed for the chromatographic identification of nonpolar steroids showed the absence of residual radioactive Δ^6-steroids, whereas all the radioactivity was concentrated at the bottom of the plates where the peptide is retained.

The peptide mixture obtained after cleavage with endoproteinase Glu-C of SHBG photolabeled with Δ^6-[^{3}H]T was also purified by reverse-phase HPLC on a C_{18} column, using an aqueous acetonitrile gradient in acidic conditions (0.1% TFA), which led to a single radioactive peptide.

Edman Sequencing of Photoaffinity-Labeled Peptides. The sequence determination of the purified peptide labeled with Δ^6-[^{3}H]E_2 showed a single sequence His-Pro-Ile-([^{3}H]X)-Arg, which was identical to the sequence obtained after labeling SHBG with Δ^6-[^{3}H]T (Grenot et al., 1988). This sequence, in which no known amino acid residue could be identified at the fourth cycle (Table II), corresponds to the pentapeptide 136–140 of SHBG sequence (Walsh et al., 1986). In both cases, the major peak of radioactivity was eluted at the fourth cycle of Edman degradation of the pentapeptide, thus suggesting specific labeling of the Met-139 amino acid residue by both reagents. A control experiment was made with the radioactive pentapeptide obtained after tryptic cleavage of SHBG photolabeled with Δ^6-[4-^{14}C]T. Edman sequencing of the peptide showed that the major peak of radioactivity was also eluted at the fourth cycle, corresponding to the undetectable transformed methionine residue.

The major peak of radioactivity released at the fourth cycle in the effluent corresponding to the elution of the undetected PTH derivative of modified methionine contained only 18–25% of the total radioactivity of the pentapeptide applied to the sequencer. This low yield seems mostly due to the great losses of radioactivity that occurred in the preceding sequencing steps, with similar patterns for the three peptides. The major part of these losses was released in the washes performed after the coupling reaction with phenyl isothiocyanate, in decreasing amounts at each cycle. Losses of radioactivity were also observed in the washing step performed at the end of the third cycle, after elution of the PTH derivative of I, as well as in the effluents containing the four PTH derivatives of H, P, I, and R. The larger amount of radioactivity that appeared at the level of the PTH derivative of R, as compared with the radioactivity that could be expected from the decreasing values observed for the PTH derivatives of H, P, and I, might correspond to the presence of an additional amount of the radioactive PTH derivative of the modified methionine residue formed at the fourth cycle, as suggested by the detection in each of the Edman degradation cycles 2, 3, 4, and 6 of a significant percentage of the PTH derivative measured in the preceding cycle. No residual radioactivity was found on the filter.

The picomoles of the missing methionine amino acid residue could be estimated from the specific activity of the photolabeling steroids and from the radioactivity eluted at the fourth cycle of Edman degradation of the corresponding photolabeled pentapeptides, assuming that one mole of steroid is attached per mole of peptide (as shown below by mass spectrometry). In all three cases these calculated values were lower but still in agreement with the mean values measured for the preceding PTH amino acids in the sequence, thus suggesting that the radioactive photolabels were covalently attached to the methionine. However, owing to the low yields of radioactivity that were recovered at the fourth Edman cycle, the possibility remains that there may be other sites of modification.

Edman sequencing of the first 24 amino acid residues of the N-terminal part of the radioactive peptide obtained after cleavage with endoproteinase Glu-C of SHBG photolabeled with Δ^6-[^{3}H]T showed that this segment corresponded to the Val-121–Gly-144 part of the human SHBG sequence. The major peak of radioactivity of this peptide was eluted at the 19th cycle of Edman degradation corresponding to Met-139.

Amino Acid Analysis. Amino acid analysis of the acid hydrolysate of an aliquot of the sample of purified Δ^6-[^{3}H]T steroid–peptide conjugate (1 nmol, estimated from the specific activity of the steroid) submitted above to Edman degradation showed the presence of 0.98–1.02 nmol (mean values of three experiments) of the four H, P, I, and R amino acids, whereas no trace of methionine could be detected. The absence of any other detectable amino acid residues confirms the purity of the radioactive pentapeptide, while the absence of methionine suggests that this peptide contains probably no significant amounts of label covalently attached to any one of the four H, P, I, and R residues, since in such a case methionine should

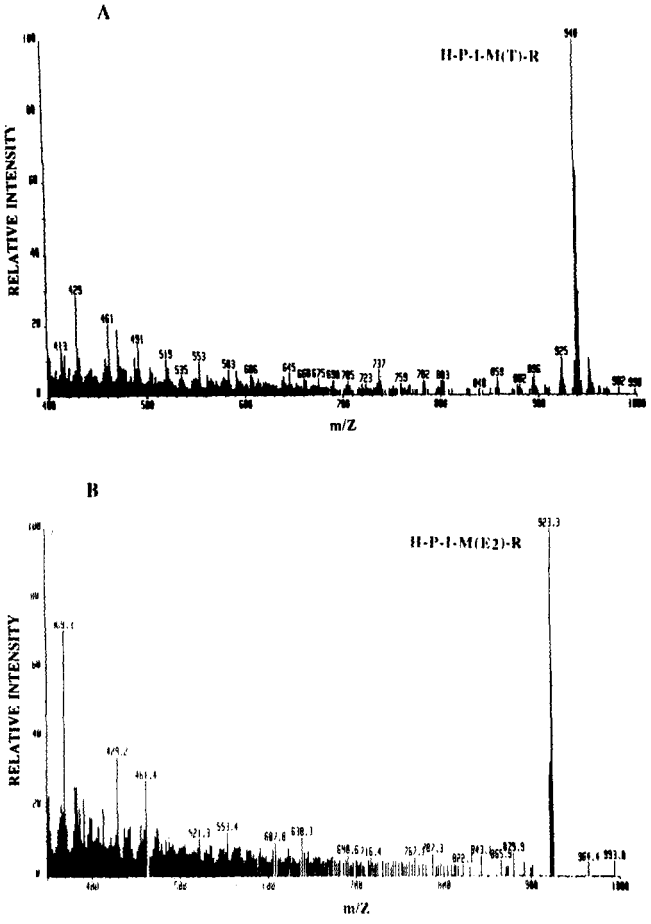

FIGURE 7: Liquid secondary ion mass spectrometry. (A) Mass spectrum of the Δ^6-T–HPI(M)R peptide. (B) Mass spectrum of the Δ^6-E_2–HPI(M)R peptide.

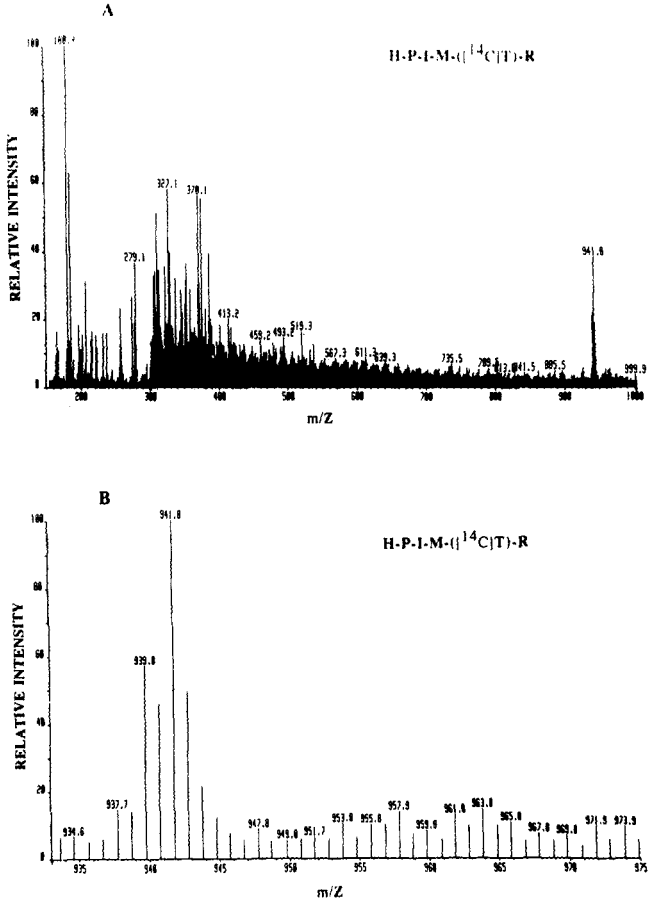

FIGURE 8: Liquid secondary ion mass spectrometry. (A) Mass spectrum of the Δ^6-[^{14}C]T–HPI(M)R peptide photolabeled with a 1:1 mixture of Δ^6-[^{14}C]T and Δ^6-T. (B) Enlargement of the zone between m/z 920 and 975.

not be modified. Moreover, no radioactivity was found in the peaks of the phenylthiocarbamyl derivatives of these four amino acids in the HPLC separation, thus excluding a contamination of these compounds by radioactive residues formed from the labeled amino acid or by tritium exchange.

Immunodetection of Photoattached Steroids on Photoaffinity-Labeled Peptides. The Δ^6-[^{3}H]T–peptide conjugate was found to bind to anti-7-*O*-(carboxymethyl)oximino-DHT antibodies (anti-DHT antibodies with 59% cross-reaction with T) but not to anti-6-*O*-(carboxymethyl)oximino-E_2 antibodies and could be displaced by an excess of DHT but not by an excess of E_2. Similarly, the Δ^6-[^{3}H]E_2–peptide conjugate was found to bind specifically to the latter anti-E_2 antibodies but not to anti-DHT antibodies and could be displaced by an excess of E_2 but not by an excess of DHT.

On the other hand, the same anti-DHT and anti-E_2 antibodies failed to recognize specifically the two corresponding photoattached radioactive steroids on uncleaved SHBG whether on nondenatured or $NaDodSO_4$–denatured SHBG.

Mass Spectrometry. To confirm the identity of the covalently attached steroid photolabels, the molecular masses of the two steroid–peptide conjugates were determined by liquid secondary ion mass spectrometry. Wide-scan mass spectra (Figures 7 and 8) were recorded at a resolution of 2000 with purified steroid–peptide conjugates in which the radioactive Δ^6-[^{3}H]T or Δ^6-[^{3}H]E_2 tracers represented less than 0.1% of the total mass of the corresponding steroid. The characteristic protonated $[M + H]^+$ ions corresponding to the Δ^6-[^{3}H]T– and Δ^6-[^{3}H]E_2–HPI(M)R conjugates appeared as the most prominent peaks at m/z 939.8 and 923.3, respectively, and corresponded well to the calculated $[M + H]^+$ molecular ion values 939.5 and 923.5, since only very low percentages of tritiated isotopes were present in labeled samples. The assignment of these two molecular ion peaks was further corroborated by recording the mass spectrum of a control sample containing all reagents except the steroid–peptide conjugate. In these latter conditions, no significant peak was present in the region m/z 900–950, where the preceding mass peaks were observed (data not shown). The molecular ion values of the two steroid–peptide conjugates indicate that the photochemical attachment corresponded in both cases to an addition of the steroid to Met-139 without any loss of mass either from the steroid or from the amino acid residue.

The presence of steroids covalently attached to the HPI-(M)R peptide was also confirmed by the comparison of mass spectra of the two Δ^6-[^{3}H]T– and Δ^6-[^{14}C]T–peptide conjugates. The Δ^6-[^{14}C]T–peptide conjugate was prepared by irradiation experiments performed with radioactive Δ^6-[^{14}C]T reagent containing nonradioactive Δ^6-T (~50%), in order to obtain an internal standard for facilitating the assignments of mass spectra. The mass spectrum of this Δ^6-[^{14}C]T–peptide conjugate (Figure 8) contained two molecular ion peaks at m/z 939.8 and 941.8, corresponding to the photoaddition of nonradioactive Δ^6-T and radioactive Δ^6-[4-^{14}C]T to the HPI(M)R peptide. The difference of 2 mass units between the two more intense mass peaks and the similarity in their relative intensities, after correction for the apparent increase of the intensity of the ion peak at m/z 941.8 due to the superimposition of the $[M + H + 2]^+$ peak associated with

the ion peak at m/z 939.8, were in good agreement with the presence of ~50% of ^{14}C isotope in the photolabeling reagent.

DISCUSSION

In this paper, we report the identification of the same Met-139 as a probable site of covalent labeling of human SHBG by two different androgenic and estrogenic photoaffinity labeling reagents, Δ^6-[^{3}H]T and Δ^6-[^{3}H]E_2.

The site-specific character of the photoaffinity labeling of SHBG with the two Δ^6-[^{3}H]T and Δ^6-[^{3}H]E_2 reagents is shown by the parallelism of the time course of covalent incorporation of each of the two radioactive Δ^6-[^{3}H]T and Δ^6-[^{3}H]E_2 photoaffinity labeling reagents with the time course of photoinactivation by the corresponding radioinert reagent and with the extent of photolysis of the steroid photolabels alone as well as by the almost total inhibition of photolabeling in the presence of an excess of DHT. The difference in the extent of specific incorporation of the two Δ^6-[^{3}H]T (0.50 mol of label/mol of SHBG) and Δ^6-[^{3}H]E_2 (0.33 mol of label/mol of SHBG) photoreagents is in accordance with the decreasing values of the corresponding association constants of T and E_2 with SHBG (Shanbag & Södergard, 1986). The dependence of the labeling efficiency on the binding affinity of the photoaffinity labeling reagents for the site was shown by the absence of photoinactivation of SHBG by the two Δ^6-T acetate and Δ^6-progesterone ligands, which have the same dienone chromophore as Δ^6-T but exhibit a much lower affinity for SHBG, and by the very low percentage of covalent labeling when photoaffinity labeling experiments were performed with Δ^6-[^{14}C]T acetate or on heat-denatured SHBG. The labeling efficiency may be limited by the rapid dissociation of steroid ligands reversibly bound to SHBG, even at low temperatures (Heyns & DeMoor, 1971; Lata et al., 1980). On the other hand, the covalent bonds, once formed, resisted well to prolonged irradiation times as shown by the absence of loss of specific covalently bound radioactivity when irradiation times were extended beyond 45 min.

The localization of the covalently bound radioactivity on SHBG was made by reverse-phase HPLC separation of radioactive peptides from tryptic digests obtained after citraconylation of lysine residues, reduction, and carboxymethylation of labeled SHBG. In all cases, most of the covalently bound radioactivity was found to be concentrated in the same major peak, whereas the rest of the covalently bound radioactivity was scattered among a multitude of different peaks of much lower intensities which were not further studied. These secondary peaks were considered as corresponding to nonspecific radioactivity, since their intensity continued to augment with the concentration of photoaffinity labeling reagents beyond the point where the intensity of the major peak ceased to increase. The two radioactive peptides corresponding to each of the two Δ^6-[^{3}H]T and Δ^6-[^{3}H]E_2 photoaffinity labeling reagents had identical retention times in the above-described HPLC separations but were unambiguously distinguished by capillary electrophoresis. These two labeled peptides, in contrast to the uncleaved labeled SHBG, were found to bind to the corresponding anti-DHT or anti-E_2 antibodies and could be displaced only by DHT in the first case or by E_2 in the second case, thus suggesting that the androgenic and estrogenic structural features of the two photoaffinity labeling reagents were probably maintained after covalent photoattachment.

Edman gas-phase sequencing of the two purified radioactive peptides corresponding to each of the two Δ^6-[^{3}H]T and Δ^6-[^{3}H]E_2 photoaffinity labeling reagents showed in both cases the same His-Pro-Ile-(*X)-Arg sequence as that reported in our preliminary communication (Grenot et al., 1988) using the Δ^6-[^{3}H]T reagent only. This sequence was easily identified as the fragment between His-136 and Arg-140 of the reported SHBG sequence, indicating, therefore, that the missing amino acid at the fourth cycle of Edman degradation corresponded to residue Met-139. The elution of the major peak of radioactivity at this fourth cycle suggests that Met-139 is the main site of photoaffinity labeling by the two Δ^6-[^{3}H]T and Δ^6-[^{3}H]E_2 reagents. A control experiment was made with the Δ^6-[^{14}C]T photoaffinity labeling reagent which demonstrated that the radioactivity measured at the elution cycle of methionine corresponded effectively to the presence of the labeled steroid and not to artifactual labeling due to tritium exchange with degradation products of methionine which could be formed during photolysis (Galardy et al., 1974) or biochemical treatments. This site of labeling is also confirmed by the comparison between the picomoles of methionine estimated from the radioactivity measured at the fourth cycle of Edman degradation and the picomoles of the preceding amino acid calculated from the corresponding PTH derivatives, which correspond in magnitude for the three labeled pentapeptides. However, the distribution of the radioactivity in the effluents of the different steps of Edman sequencing of all three peptides (Table II) indicates that the relatively low yields of radioactivity (18–25% of the total radioactivity of the pentapeptide applied to the sequencer) measured at the fourth cycle are mainly the consequence of losses of radioactivity probably due, for their greatest part, to the hydrophobic character conferred by the attachment of a large steroid molecule to a short peptide. Amino acid analysis of the acid hydrolysate of the purified Δ^6-[^{3}H]T–HPI(M)R peptide gave quantitative yields of H, P, I, and R amino acids that render very improbable the presence of a peptidic contaminant totally resistant to Edman sequencing, i.e., by photodegradation, and undetectable by chromatography and mass spectrometry. On the other hand, the total absence of methionine is an argument against the formation of an isomeric radioactive peptide with an acid-labile covalent attachment of the steroid to one of the four other amino acids that should have resulted in the presence of a corresponding amount of unmodified methionine residue. Another argument for the location of radioactive labeling on Met-139 is the elution of the major peak of covalently bound radioactivity at the corresponding 19th cycle of Edman degradation of the Val-121–Gly-144 fragment of the peptide obtained after cleavage with endoproteinase Glu-C of SHBG photolabeled with Δ^6-[^{3}H]T, which shows also that covalent attachment of Δ^6-[^{3}H]T is relatively resistant to the conditions of sequencing.

The structure of the two tritiated steroid–pentapeptide conjugates was confirmed by liquid secondary ion mass spectrometry, which showed $[M + H]^+$ peaks at m/z 939.8 and 923.3, corresponding exactly to the addition of Δ^6-T and of Δ^6-E_2 photoaffinity labeling reagents to the HPI(M)R peptide. The photoaddition of the Δ^6-T steroid molecule was corroborated by the difference of 2 mass units between the $[M + H]^+$ peaks at m/z 939.8 and 941.8 observed when SHBG was irradiated in the presence of Δ^6-T containing the Δ^6-[4-^{14}C]T tracer. Therefore, these molecular weights indicate that neither the steroid nor the peptide moiety had suffered from the above-mentioned degradation processes.

Edman sequencing, amino acid analyses, and mass spectrometry experiments strongly suggest that Met-139 is the major site of photolabeling by the three tested Δ^6 steroids, although these data provide only indirect evidence. On the

other hand, the fraction of the total radioactivity recovered in the major peak collected at the fourth cycle of Edman degradation of the three corresponding steroid–HPI(M)R conjugates is too low to exclude unequivocally the possibility of other sites of covalent fixation. Further investigations remain necessary to establish the structures of the covalent amino acid–steroid adducts and to determine whether these conjugates are linked at the same positions of the steroid molecule and of the amino acid side chain.

Little is known about the mechanism of photoaffinity labeling with the Δ^6-T ligand as well as with other related dienone and trienone reagents. The addition of double bonds to extend the carbonyl conjugation of Δ^4-3-ketone chromophores produces a shift of the n–π^* transition from 305 to 345 nm (Taylor et al., 1980a), far beyond the upper limit of protein absorption. Such a shift facilitates the use of excitation wavelengths above 300 nm, which limits the irreversible photodenaturation of the protein. It is generally well-admitted (Benisek, 1977; Gronemeyer & Govindan, 1986; Sweet & Murdock, 1987) that UV irradiation excites the conjugated ketone to a short-lived singlet state which is rapidly converted by intersystem crossing to a long-lived n–π^* triplet state ($\sim 10^{-3}$ s at 77 K). This excited triplet state has a diradical character and is thought to operate hydrogen abstraction from the side chain of an amino acid residue of the protein by the carbonyl oxygen atom of the photoexcited chromophore, whereas no reaction occurs with water, owing to the low energy of this triplet. Other mechanisms could also play a role, depending on the structural characteristics of the binding site. As an example, it has been proposed that the less reactive π–π^* triplet state could be involved in the formation of a saturated 4-benzyl derivative after irradiation of T acetate in toluene as a result of the abstraction of a benzylic hydrogen from the toluene molecule by the β-carbon of the enone chromophore (Bellus et al., 1969).

The covalent attachment of Δ^6-[^{3}H]E_2 might also proceed through a hydrogen abstraction from the methionine side chain, although this mechanism does not appear among those proposed for the interpretation of the photochemical reactivity of styrene chromophores in solution (Kropp, 1979). However, such a mechanism could be favored in the environment of the binding site (Bayley & Staros, 1984). On the other hand, the hypothesis of labeling mechanisms based on the reactivity of Δ^6-E_2 either as active-site sensitizer of photooxidation (Knowles, 1972) or as photooxidation substrate (Kropp, 1979; Planas et al., 1989a,b), is very improbable since all traces of oxygen were eliminated by careful degassing of SHBG samples and by the use of an inert argon atmosphere during photoaffinity labeling experiments, conditions which were found to improve the yields of photoaffinity labeling. The progressive character of the incorporation of radioactivity in the two steroid–HPI(M)R peptides with increasing irradiation times suggests that the unique location of specific covalent photoattachment of Δ^6-[^{3}H]T or Δ^6-[^{3}H]E_2 corresponds probably, in each case, to a single photoprocess operating from the beginning to the end of irradiation time. The absence of modifications of the labeled peak and the absence of formation of progressively increasing secondary peaks after prolonged irradiation times (up to 1 h) show the photochemical stability of the covalent bonds once formed. Further investigations on the structure of the two steroid–peptide covalent conjugates will help to determine the nature of the photochemical processes involved in their formation.

The labeling of Lys-134 and Met-139 residues is in agreement with predictions based on sequence correlations between different steroid metabolizing enzymes, steroid receptors, and steroid binding proteins, which showed a consensus sequence located between residues 112 and 128 of SHBG or residues 142 and 158 of rat ABP (Picado-Leonard & Miller, 1988). However, the general validity of these predictions has been recently questioned since deletion of this consensus sequence from estradiol receptors was shown to have no effect on steroid binding activity (Fawell et al., 1989). The two Lys-134 and Met-139 amino acids are also located at positions very close to the hydrophobic segment 141–149 of human SHBG (Walsh et al., 1986). These two amino acid residues are both present in the two sequences established for human SHBG (Hammond et al., 1987; Que & Petra, 1987; Gershagen et al., 1987) and rabbit SHBG (Griffin et al., 1989). However, the sequences of rat (Joseph et al., 1987), mouse, or rabbit ABPs (Wang et al., 1989) also contain a methionine at a position (Met-169) equivalent to that of Met-139 of SHBG, whereas only rabbit ABP displayed a lysine at the position corresponding to Lys-134 of SHBG. The importance of these two amino acids, as well as that of other amino acid residues present in the environment of the hydrophobic segment 141–149 of human SHBG, for the specific recognition of androgen and estrogen ligands remains to be established by sequence analyses of SHBGs and ABPs from other species and by experiments of mutagenesis.

The reactivity of Lys-134 with a 17β-bromoacetoxy group and the photoaffinity labeling of Met-139 with a $\Delta^{4,6}$-3-dienone chromophore suggest that the Lys-134 residue lies in the immediate vicinity of the 17-OH group, whereas Met-139 should lie near rings A or B at the other end of the steroid molecule. This peptide segment would therefore delineate a large part of the site around the steroid label, provided that the two labeled residues belong to the same subunit and that affinity labeling reagents retain the same position in the binding site as unmodified ligands. However, the presence of a bromoacetate substituent at the C-17 position, which confers a low affinity to the nucleophilic DHT reagent, as well as the possible rotation of the bromoacetoxy group around the 17,20 single bond (Holmes & Smith, 1983) can both facilitate a shift of the entire reagent toward the nearest reactive residue available around the site. On the other hand, the ability of human SHBG to accomodate the three DHT, T, and E_2 ligands as well as 2-methoxy-E_2 (Dunn et al., 1980) or 2-iodo-E_2 (Fernlund & Gershagen, 1990), each with different A-ring structures, suggests a loose interaction with the peptide segments corresponding to rings A or B. Conversely, the structures of rings C and D, which are common to the five latter ligands, could be expected to correspond to a more selective interaction. However, the ability of the SHBG binding site to accomodate 17-oxo steroids such as E_1, 2-methoxy-E_1 (Philip & Murphy, 1986a,b), or equilenin (Orstan et al., 1986) does not indicate a very high specificity for the 17 substituent either.

As long as the mechanisms of photoaffinity labeling are not known, one can only speculate whether the selective photoaffinity labeling of the same Met-139 results from a similar rigid positioning of the two Δ^6-T and Δ^6-E_2 photoaffinity reagents or if the labeling of methionine results from an enhanced reactivity of this residue toward active photoexcited species having sufficiently long lifetimes. In this latter case, the formation of a covalent bond with Met-139 could occur in the immediate vicinity of the hydrophobic IALGGLLFP 141–149 peptide segment without meaning that the labeled amino acid is involved in the primary binding process. However, the recent photolabeling of the IAL-

GGLLLPTS 171–181 peptide segment of rat ABP with Δ^6-T does not indicate the presence of radioactivity on the Met-169 residue of the rat ABP precursor, which corresponds to Met-139 of SHBG (Danzo et al., 1991). Other examples of a covalent labeling of methionine residues are the photoaffinity labeling of the glucocorticoid receptor from rat liver cytosol and of the human glucocorticoid receptor by two different reagents, [^{3}H]triamcinolone acetonide and [^{3}H]promegestone (R5020), which resulted in the selective labeling of the same Met-622 and Cys-754 amino acids in the rat receptor and of the corresponding Met-604 and Cys-736 residues in human receptor (Carlstedt-Duke et al., 1988; Strömstedt et al., 1990), whereas the photoaffinity labeling of human progestin receptors by [^{3}H]promegestone (R5020) resulted in the labeling of two Met-759 and Met-909 residues (Strömstedt et al., 1990). Determining whether the two Δ^6-[^{3}H]T and Δ^6-[^{3}H]E_2 photoaffinity labeling reagents are linked at similar positions of the steroid molecule and of the labeled amino acid will help to define the positioning of these two reagents in the binding site of SHBG.

ADDED IN PROOF

A very recent paper (Bocchinfuso et al., 1992) reports site-directed mutagenesis experiments on hSHBG that resulted in a marked reduction of the steroid-binding affinity of a Trp-139 mutant, whereas two other Asp-133 or Gln-136 mutants showed normal binding properties. In that work, a chimera containing the N-terminal portion of hSHBG (205 residues) and the C-terminal portion of rat ABP was found to display similar binding properties of hSHBG, thus confirming the location of the steroid-binding site in the N-terminal portion of hSHBG.

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REFERENCES

Bayley, H. (1983) in *Photogenerated Reagents in Biochemistry and in Molecular Biology* (Work, T. S., & Burdor, R. H., Eds.) pp 107–111, Elsevier, Amsterdam.

Bayley, H., & Staros, J. V. (1984) in *Azides and Nitrenes* (Scriven, E. F. V., Ed.) pp 433–490, Academic Press, Orlando, FL.

Bellus, D., Kearns, D. R., & Schaffner, V. (1969) *Helv. Chim. Acta 52*, 971–1009.

Benisek, W. F. (1977) *Methods Enzymol. 46*, 469–479.

Bocchinfuso, W. P., Warmels-Rodenhiser, S., & Hammond, G. L. (1992) *FEBS Lett. 301*, 227–230.

Carlstedt-Duke, J., Strömstedt, P.-E., Persson, B., Cederlund, E., Gustafsson, J.-A., & Jörnvall, H. (1988) *J. Biol. Chem. 263*, 6842–6846.

Casali, E., Petra, P. H., & Ross, J. B. A. (1990) *Biochemistry 29*, 9334–9343.

Cheng, C. Y., Musto, N. A., Gunsalus, G. L., & Bardin, W. C. (1983a) *J. Steroid Biochem. 19*, 1379–1389.

Cheng, C. Y., Bardin, W. C., Musto, N. A., Gunsalus, G. L., Cheng, S.-L., & Ganguly, M. (1983b) *J. Clin. Endocr. Metab. 56*, 68–75.

Cheng, C. Y., Musto, N. A., Gunsalus, G. L., Frick, J., & Bardin, W. C. (1985) *J. Biol. Chem. 260*, 5631–5640.

Cheng, S.-L., Kotite, N., & Musto, N. A. (1984) *J. Steroid Biochem. 21*, 669–676.

Danzo, B. J., Taylor, C. A., Jr., & Eller, B. C. (1982) *Endocrinology 111*, 1278–1285.

Danzo, B. J., Parrott, J. A., & Skinner, M. K. (1991) *Endocrinology 129*, 690–696.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.

Dunn, J. F., Merriam, G. R., Eil, C., Kono, S., Loriaux, D. L., & Nisula, B. C. (1980) *J. Clin. Endocr. Metab. 51*, 404–406.

Englebienne, P., Van Hoorde, P., & Verheyden, R. (1987) *J. Steroid Biochem. 26*, 527–534.

Fawell, S. E., Lees, J. A., & Parker, M. G. (1989) *Mol. Endocrinol. 3*, 1002–1005.

Fernlund, P., & Gershagen, S. (1990) *J. Steroid Biochem. 36*, 75–81.

Galardy, R. E., Craig, L. C., Jamieson, J. D., & Printz, M. P. (1974) *J. Biol. Chem. 249*, 3510–3518.

Gershagen, S., Fernlund, P., & Lundvall, A. (1987) *FEBS Lett. 220*, 129–135.

Glazer, A. N., Delange, R. J., & Sigman, D. S. (1975) in *Chemical Modification of Proteins* (Work, T. S., & Work, E., Eds.) pp 79–84, Elsevier Biomedical Press, Amsterdam.

Grenot, C., & Cuilleron, C. Y. (1979) *Steroids 34*, 15–34.

Grenot, C., De Montard, A., Blachère, T., Mappus, E., & Cuilleron, C. Y. (1988) *C. R. Acad. Sci., Ser. 3 307*, 391–396.

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.

Gronemeyer, H., & Govindan, M. V. (1986) *Mol. Cell. Endocrinol. 46*, 1–19.

Hammond, G. L. (1990) *Endocr. Rev. 11*, 65–79.

Hammond, G. L., Langley, M. S., & Robinson, P. A. (1985) *J. Steroid Biochem. 23*, 451–460.

Hammond, G. L., Underhill, D. A., Smith, C. L., Goping, I. S., Harley, M. J., Musto, N. A., Cheng, C. Y., & Bardin, C. W. (1987) *FEBS Lett. 215*, 100–104.

Heukeshoven, J., & Dernick, R. (1985) *Electrophoresis 6*, 103–112.

Heyns, W., & DeMoor, P. (1971) *J. Clin. Endocr. Metab. 32*, 147–154.

Holmes, S. D., & Smith, R. G. (1983) *Biochemistry 22*, 1729–1734.

Joseph, D. R., Hall, S. H., & French, F. S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 339–343.

Katzenellenbogen, J. A., Johnson, H. J., Jr., Carlson, K. E., & Myers, H. N. (1974) *Biochemistry 13*, 2986–2994.

Khan, M. S., & Rosner, W. (1990) *J. Biol. Chem. 265*, 8431–8435.

Knowles, J. R. (1972) *Acc. Chem. Res. 5*, 155–160.

Kropp, P. J. (1979) in *Organic Photochemistry* (Padma, A., Ed.) Vol. 4, pp 2–142, Marcel Dekker, New York.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lata, G. F., Hu, H.-K., Bagshaw, G., & Tucker, R. F. (1980) *Arch. Biochem. Biophys. 199*, 220–227.

Mickelson, K. E., & Petra, P. H. (1974) *FEBS Lett. 44*, 34–38.

Mickelson, K. E., & Petra, P. H. (1978) *J. Biol. Chem. 253*, 5293–5298.

Namkung, P. C., Kumar, S., Charbonneau, H., & Petra, P. H. (1988) *Steroids 52*, 333–334.

Namkung, P. C., Kumar, S., Walsh, K. A., & Petra, P. H. (1990) *J. Biol. Chem. 265*, 18345–18350.

Orstan, A., Lulka, M. F., Eide, B., Petra, P. H., & Ross, J. B. A. (1986) *Biochemistry 25*, 2686–2692.

Payne, D. W., Katzenellenbogen, J. A., & Carlson, K. E. (1980) *J. Biol. Chem. 255*, 10359–10367.

Petra, P. H. (1991) *J. Steroid Biochem. 40*, 735–753.

Petra, P. H., Kumar, S., Hayes, R., Ericsson, L. H., & Titani, K. (1986a) *J. Steroid Biochem. 24*, 45–49.
Petra, P. H., Namkung, P. C., Senear, D. F., McCrae, D. A., Rousslang, K. W., Teller, D. C., & Ross, J. B. A. (1986b) *J. Steroid Biochem. 25*, 191–200.
Petra, P. H., Que, B. G., Namkung, P. C., Ross, J. B. A., Charbonneau, H., Walsh, K. A., Griffin, P. R., Shabanowitz, J., & Hunt, D. F. (1988) *Ann. N.Y. Acad. Sci. 538*, 10–24.
Philip, A., & Murphy, B. E. P. (1986) *Steroids 47*, 373–379.
Philip, A., & Murphy, B. E. P. (1989) *J. Steroid Biochem. 32*, 873–885.
Picado-Leonard, J., & Miller, W. L. (1988) *Mol. Endocrinol. 2*, 1145–1150.
Planas, A., Lupon, P., Cascallo, M., & Bonet, J.-J. (1989a) *Helv. Chim. Acta 72*, 715–724.
Planas, A., Sala, N., & Bonet, J.-J. (1989b) *Helv. Chim. Acta 72*, 725–730.
Que, B. G., & Petra, P. H. (1987) *FEBS Lett. 219*, 405–409.
Renoir, J.-M., Mercier-Bodard, C., & Baulieu, E.-E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4578–4582.
Rosner, W. (1990) *Endocr. Rev. 11*, 80–91.
Scatchard. G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660-672.
Shanbag, V. P., & Södergard, R. (1986) *J. Steroid Biochem. 24*, 549–555.
Strel'Chyonok, O. A., & Avvakumov, G. V. (1990) *J. Steroid Biochem. 35*, 519–534.
Strömstedt, P.-E., Berkenstam, A., Jörnvall, H., Gustafsson, J.-A., & Carlstedt-Duke, J. (1990) *J. Biol. Chem. 265*, 12973–12977.
Sweet, F., & Murdock, G. L. (1987) *Endocr. Rev. 8*, 154–184.
Taylor, C. A., Jr., Smith, H. E., & Danzo, B. J. (1980a) *Proc. Natl. Acad. Sci. U.S.A. 77*, 234–238.
Taylor, C. A., Jr., Smith, H. E., & Danzo, B. J. (1980b) *J. Biol. Chem. 255*, 7769–7773.
Turner, A. B., & Ringold, H. J. (1967) *J. Chem. Soc. C*, 1720–1730.
Walsh, K. A., Titani, K., Takio, K., Kumar, S., Hayes, R., & Petra, P. H. (1986) *Biochemistry 25*, 7584–7590.
Wang, Y.-M., Sullivan, P. M., Petrusz, P., Yarbrough, W., & Joseph, D. R. (1989) *Mol. Cell. Endocrinol. 63*, 85–92.