Identification of Lysine 134 in the Steroid-binding Site of the Sex Steroid-binding Protein of Human Plasma*

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The sex steroid-binding protein of human plasma SBP (or sex hormone-binding globulin, SHBG) was specifically inhibited with the alkylating affinity label, 17β-[(2-14C)bromoacetyl]oxy]-5α-androstan-3-one. The natural ligand, 5α-dihydrotestosterone, was shown to protect against inactivation and labeling. The steroid-binding activity of the protein was abolished when approximately 1 mol of label was incorporated into 1 mol of dimeric SBP. In order to identify and locate the labeled amino acid in the steroid-binding site, the steroidal portion of the bound label was first removed and the protein was digested with Achromobacter protease and subdigested with trypsin. Seven radioactive peptides were isolated, sequenced, and found to contain the common sequence QVSGPLTSXR. Residue X was identified as lysine-134 from the SBP amino acid sequence (Walsh, K. A., Titani, K., Kumar, S., Hayes, R., and Petra, P. H. (1986) Biochemistry 25, 7584–7590). The results indicate that only 1 of the 2 lysine-134 residues in the homodimer was labeled. This suggests that the steroid-binding site is constructed from an association of the two subunits in an AB to BA “sandwich” configuration with lysine-134 residue of one subunit on one surface near the D-ring and the lysine-134 of the other subunit at the opposite end of the steroid, or well away from the steroid-binding site. Although the nature of the data does not allow description of a specific role for lysine-134, its proximity to the 17β-OH of the steroid nucleus suggests participation in the binding process through direct or indirect hydrogen bonding.

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The abbreviations used are: SBP, plasma sex steroid-binding protein; SHBG, sex hormone-binding globulin; DHT, 5α-dihydrotestosterone; T, testosterone; DHTBr, 17β-[(bromoacetyl)oxy]-5α-androstan-3-one; [3H]DHTBr, 17β-[(3H)bromoacetyl]oxy]-5α-androstan-3-one; [14C]DHTBr, 17β-[(2-14C)bromoacetyl]oxy]-5α-androstan-3-one; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IAA, iodoacetate; HPLC, high performance liquid chromatography.

EXPERIMENTAL PROCEDURES

Materials—Human pregnancy serum was obtained from Dr. Steve Plymate at the Madigan Army Hospital in Tacoma, WA. 5α-Dihydrotestosterone was purchased from Steraloids. Anhydrous methylene chloride was obtained from Aldrich. Bromoacetic acid, 1,3-dicyclohexylcarbodiimide, and crystalline bovine serum albumin were from Sigma. Silica Gel G was purchased from Brinkmann Instruments. Bromo-2,4-dichloroacetic acid (56 μCi/mmol) was purchased from Amer sham and [1,2-3H]DHT (58.4 Ci/mmol) from DuPont-New England Nuclear. DEAE-cellulose filter paper discs (DE81, 2.3-cm diameter) were purchased from Roche Angel Co. Aqueous-2 was purchased...
Purification of Human SBP—SBP was purified to homogeneity as previously described (Petra et al., 1986a) and M, = 93,400 (Petra et al., 1986b).  

Assay of SBP Activity—The DEAE-cellulose filter assay previously described for measuring steroid-binding proteins in plasma (Michelson and Petra, 1974; Schiller and Petra, 1975) was used with some modification. Aliquots from the DHTBr reaction vessel (20 μl containing about 2 μg of SBP) were added to 300 μl of bovine serum albumin solution followed by a 30-min incubation at 25 °C. These were then added to 0.5 ml of charcoal (0.5% charcoal, 0.05% dextran (w/v), 0.1% gelatin, 10 mM Tris-Cl, pH 7.4). The suspensions were shaken gently for 15 min at room temperature and centrifuged at 4 °C for 5 min at 18,000 × g to remove charcoal-containing steroid. Aliquots were further diluted 10-fold with 10 mM Tris-Cl, pH 7.4, and incubated for 20 min at room temperature with a 3-fold molar excess of [3H]DHT over SBP. The solutions were then added to 1.5 mg of pellets obtained from 0.5% charcoal, 0.05% dextran, 0.1% gelatin, 10 mM Tris-Cl, pH 7.4. The suspensions were further incubated for 20 min at 0 °C, and 50 μl aliquots were applied to a stack of two DEAE-filter paper discs for assay.

Synthesis of 17α-(Bromoacetyloxy)5α-androstan-3-one—Synthesis was carried out according to published procedures (Sweet and Samanta, 1980) starting with 0.1 mmol of DHT, bromoacetic acid, and 1,3-dicyclohexylcarbodiimide. After mixing the reagents in 4 ml of anhydrous CH2Cl2, the reaction was allowed to proceed in 2 ml of CH2Cl2. The same reaction conditions were used after the affinity chromatography and antitransferrin immunoprecipitations. The structure of DHTBr is shown in Fig. 1B. Radioactive [3H]DHTBr (specific activity 1.23 μCi/μmol) and radioactive DHTBr were synthesized according to published procedures (Takio et al., 1983). After dialysis against 0.1 M NaHCO3, 10 mM phosphate, pH 6, at 1 ml/min. Fractions were monitored at 226 nm, and 5% of each were analyzed for 3H. Radioactive peptide fractions were pooled separately and fractionated by reverse phase HPLC on a Beckman Ultrapure RPSC column (7-μ C3, 4.6 × 75 mm) using an acetonitrile/water gradient in 0.08% trifluoroacetic acid. Tryptic subdigests were done in 0.1 M NH4HCO3 and fractionated on a column of RP300/102 (7-μ C8, 2.1 × 100 mm). Peptide purity was checked by amino acid analysis.

Gel Electrophoresis—SDS-gel electrophoresis was carried out according to procedures published by Litwack et al. (1986). Native gel electrophoresis was carried out in tubes according to Petra et al. (1985).

Amino Acid Analysis and Peptide Sequence Determination—Amino acid analyses were carried out as previously published (Biddulph et al., 1984). Peptides were sequenced with an Applied Biosystems model 470 Sequencer with on-line phenylthiohydantoin analysis using published programs (Hunkapiller et al., 1983).

Mass Spectrometry—Mass spectrometry was performed at the Department of Physics, University of California, Berkeley, on a Finnigan MAT-8200 instrument. Mass spectrometric analysis was carried out in the negative ion mode.

RESULTS

Kinetics of DHTBr Labeling—The kinetics of SBP inactivation with DHTBr and incorporation of the label are shown in Fig. 1, A and B. In the presence of 10.1 molar excess of label over SBP, 50% inactivation occurs in approximately 2 h at 37 °C. During that time, about 0.5 mol of label is covalently incorporated into 1 mol of dimeric SBP. Labeling occurs in the steroid-binding site because loss of activity and label incorporation are both retarded in the presence of a 10-fold molar excess of DHT over DHTBr. Reaction of native SBP with [3H]iodoacetic acid results in no incorporation of radioactivity into the protein (data not shown) indicating that the halogen on the methyl ketone functional group must be attached to the natural ligand, as in the case of DHTBr, in order to react specifically in the steroid-binding site. Fig. 1, A and B also indicates that about 30% of the activity is lost after 4 h in the presence of DHT with a concomitant incorporation of about 30% of label into SBP.

 Stoichiometry of Label Incorporation—The distribution of radioactivity in SDS-PAGE after a 180-min reaction is shown in Fig. 2. Two radioactive peaks are detected, peak I (major) at M, = 44,000 where the denatured human SBP monomer
absence of DHT and DHTBr. Percent loss of SBP activity in the
ration into peak I of Fig. 2 as measured by SDS-PAGE, in the absence
absence (O—O) or presence (W) of DHT. B, labelling incorpo-
with DHTBr at pH 7.4 and 37 °C. [SBP] = 1
photometric determination of SBP concentrations using \( \epsilon_{390} = 1.14 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1} \).

Fig. 1. Kinetics of SBP inhibition and label incorporation
with DHTBr at pH 7.4 and 37 °C. [SBP] = 1 \( \mu \text{M} \); [DHTBr] = 10
\( \mu \text{M} \); [DHT] = 100 \( \mu \text{M} \). A, \( \Delta \ldots \Delta \), control, SBP activity in the
absence of DHT and DHTBr. Percent loss of SBP activity in the
presence (O—O) or presence (C—C) of DHT. The star in the chemical
structure of DHTBr indicates position of the \(^{14}\text{C}\) isotope. Mole of
label incorporated per mol of dimeric SBP is calculated from the
specific radioactivity of \([^{14}\text{C}]\text{DHTBr}\) (1.23 \( \mu \text{Ci/\mu mol} \)) and spectro-
photometric determination of SBP concentrations using \( \epsilon_{390} = 1.14 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1} \).

Fig. 2. Analysis of affinity-labeled SBP by SDS-PAGE. Left
panel shows radioactivity distribution in a SDS gel (2-mm slices)
derived from a 180-min reaction using the conditions described under
“Experimental Procedures” and in the legend of Fig. 1. Affinity
labeling is carried out in the absence (O—O) or in the presence
(O—O) of DHT. The right panel displays a SDS slab-gel stained
with Coomassie Blue. Lane I, 10 pg of standard proteins (Bio-Rad);
lane 2, 20 \( \mu \text{g} \) of SBP; lane 3, 40 \( \mu \text{g} \) of affinity-labeled SBP. The roman
numerals next to the \( M_r = 44,000 \) (44K) and 28,000 (28K) bands
correspond to their position in the pattern shown in the left panel.

normally migrates, and peak II (minor) at about 28,000. The
presence of DHT in the mixture lowers the radioactivity
content of both peaks. The SDS-PAGE data of Fig. 2 indicate that
about 0.8 mol of label is incorporated per mol of
dimeric SBP after 4 h; when peak II is added to peak I, the
stoichiometry reaches about 1.1 mol/mol. A stained SDS slab-
gel containing samples from a MO-min reaction and from
unreacted SBP is also shown in Fig. 2. As expected, there are
two stained bands at about 44,000 and 28,000 corresponding
to radioactive peaks I and II (lane 3). Native SBP (lane 2)
do not contain the \( M_r = 28,000 \) band.

Electrophoresis of Labeled SBP in Native Gels—In order to
determine whether or not labeling disrupts the dimeric
structure of SBP, protein was reacted with DHTBr as described
above and electrophoresed in native gels along with native
SBP. Fig. 3 shows that the labeled protein migrates at the
same place as native protein indicating that the native dimeric
structure is maintained.

Removal of Steroid from Labeled SBP—To gain further
insight into the labeling reaction, SBP was inactivated with
\([^{14}\text{C}]\text{DHTBr}\) as described above, and also with \([1,2-^3\text{H}]\)
DHTBr, which contains the label in the steroid nucleus
instead of the 17β side chain. As described above, both reactions
were exhaustively dialyzed against IAA, and against
water to remove IAA. Both reactions were then incubated at
pH 11 for 1 h at 37 °C to cleave the covalently bound steroid
through hydrolysis of the ester linkage in the acetoxy side
chain. The freeze-dried products were electrophoresed in SDS.
The gel pattern shown in Fig. 4A demonstrates that the \(^3\text{H}-
labelled steroid was removed since neither peak I nor II was
radioactive after base treatment, whereas SBP retained label
from \([^{14}\text{C}]\text{DHTBr}\) (Fig. 4B). The data further suggest that
the label is not esterified to aspartic or glutamic acid residues
because the \(^{14}\text{C}\) label remains with the protein at high pH.
These experiments show that cleavage of the acetoxy ester
bond by base removes the steroid moiety of the label, thereby
reducing the hydrophobicity of labeled peptides and facilitating
their isolation.

Identification of the Site of Affinity Labeling—Fifteen nmol
of the affinity-labeled SBP polypeptide (calculated as the
monomer, \( M_r = 46,700 \)), was dialyzed as described above,
incubated at pH 11 to remove the steroid moiety, and
released and S-carboxymethylated. After dialysis against 0.1
\( \text{M NH}_2\text{HCO}_3 \), the sample contained 50,000 cpm. Since 15 nmol
(monomeric) of labeled SBP should contain about 18,000 cpm
based on label incorporation of 1.1 mol/mol of dimeric SBP
(combined peaks I and II of Fig. 2), the data indicated that
about 32,000 cpm of unbound label was still present after
dialysis. To reduce protein losses, the labeled protein was not
dialyzed further but digested at lysyl residues and fractionated
by size (see "Experimental Procedures"). Two peptide-bound
radioactive fractions were obtained, and each was fractionated
on reverse phase HPLC C3 columns to yield four radioactive
fractions containing a total of 5,275 cpm (2.1 nmol of label,
25% yield). Preliminary analyses of small aliquots suggested
that these fractions contained overlapping products of incom-
plete digestion. The four labeled fractions were pooled and

Fig. 3. Electrophoresis of labeled and native SBP in native
polyacrylamide gels. Methods as described in Petra et al., 1983,
extcept that electrophoresis was carried out in tubes. One nmol of
SBP was reacted with a 10-fold molar excess of DHTBr as described
under "Experimental Procedures" in the absence of DHT (O—O)
or the presence of 10-fold molar excess over DHTBr (O—O). The
gels were sliced and counted. The arrow represents the position where
native SBP migrates as detected by Coomassie Blue staining.
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FIG. 4. Removal of covalently bound steroid from affinity-labeled SBP at pH 11 for 1 h at 37 °C as determined by SDS-PAGE. A, SBP labeled with [3H]DHTBr, untreated (O—O) or treated (O—O) at pH 11. B, SBP labeled with [14C]DHTBr, untreated (O—O) or treated (O—O) at pH 11.

FIG. 5. Separation of tryptic peptides from a mixture of labeled fractions derived by cleavage of affinity-labeled SBP with Achromobacter I. HPLC was carried out on a column of RP300/102 (a 7-μ C8 column, 2.1 x 100 mm) using an acetonitrile/water gradient in 0.1% trifluoroacetic acid. Hatch marks indicate pooled fractions containing label.

subdigested with trypsin, and products were separated on a C8 reverse phase HPLC column. As shown in Fig. 5, five labeled peptide fractions were recovered and 40-80% of each was subjected to Edman degradation.

The results in Table I reveal that the common sequence QVSGPLTSXR is present in each fraction, although minor contaminants are seen as well as long versions of the same peptide. The residue denoted X in the major peptides of Table I did not yield an identifiable phenylthiohydantoin but, from the amino acid sequence of human SBP (Walsh et al., 1986), that residue corresponds to lysine 134. Since all the other residues in this sequence were easily recognized, it is concluded that Lys 134 is the site of N-carboxymethylation by the affinity label, and that the phenylthiohydantoin of N-carboxymethyllysine does not elute in a readily recognized location in our chromatographic system.

Assignment of Lys-134 as the site of labeling was confirmed by time-of-flight mass spectrometry on fractions T-17 and T-20 of Table I. The major ion in T-17 had a m/z of 1400.6, corresponding to the calculated mass (1400.5) of N-carboxymethyl-LRQVSGLTSKFR. Fraction T-20 revealed two ions, of m/z 1749 and 1766. The difference in mass between these two ions corresponds to the 17 mass units of lost NH3 when glutamine cyclizes to a pyroglutamyl residue. Therefore, the two ions in T-20 correspond in mass to the N-carboxymethyl forms of QVSGLTSKFRHPIMIR and its blocked pyroglutamyl derivative. The blocked pyroglutamyl form of that peptide probably accounts for much of the label in T-22, although this was not proven by mass spectrometry.

In summary, all of these results point to Lys-134 as the site of labeling. No other minor peptide was found in more than one labeled fraction. As shown in Table I, the specific activity of the label (2500 cpm/nmol) agrees well with the amount of the major peptide observed in the Sequencer with each of the peptide preparations. The total amount of radioactivity recovered as Lys-134-containing peptides was 15–20%; this is a good recovery considering that the generally accepted peptide recovery yield per HPLC column is 50%.

DISCUSSION

Description of the chemical environment of steroid binding sites is needed for understanding the molecular basis of steroid-binding specificity. In this paper we report the presence of lysine 134 in the steroid-binding site of human SBP by virtue of its specific alkylation with the affinity label, 17β-[bromoacetyl]oxy-5α-androstan-3-one. Specificity of the labeling reaction is shown by protection with the natural ligand, 5α-dihydrotestosterone. Since incorporation of approximately 1 mol of label per mol of dimeric SBP abolishes the steroid-binding activity of the protein, inactivation is a direct consequence of Lys-134 labeling.

As shown in Fig. 1, inactivation and label incorporation also occur in the presence of DHT, although at a lower rate. Incomplete protection would occur if DHTBr were to compete efficiently with DHT, thereby displacing it in the steroid-binding site. This would result in a gradual decrease in SBP activity with time as indicated in Fig. 1. Similar observations were reported for DHTBr inactivation of the androgen receptor in prostate cytosols where protection by DHT was also found to be incomplete (Kovacks and Turney, 1988). These authors report that DHTBr binding to the receptor was only 2.3-fold lower than DHT. We have not analyzed peptides obtained from labeled SBP in the presence of DHT, but we strongly suspect that lysine 134 is also alkylated under those conditions. This is supported by the fact that only one site of modification was found when the protein was reacted in the absence of DHT. Although similar data would be expected if some labeling were to occur at nonspecific sites in addition to the steroid-binding site, this seems unlikely because label incorporation and loss of activity occur at the same rate in a 1:1 stoichiometry in the absence of DHT. Therefore, the likely explanation is that DHTBr competes efficiently with DHT for the binding site.

Fig. 1 also indicates that the rate of inhibition and label...
incorporation appears to decrease after 3 h in the presence of DHT. Although we did not carry out a complete kinetic analysis, this phenomenon is probably caused by a decrease in DHTBr concentration as reaction proceeds. This effect is less pronounced in the absence of DHT because less affinity label is required to maintain a pseudo-first order reaction. In the presence of DHT, however, more reagent is needed to maintain the rate of the reaction which is dependent upon efficient competition with DHT.

The data also indicate that labeling of only one of the two identical subunits of homodimeric SBP is sufficient to abolish the activity. The radiolabeled peptides contain only one site of modification, carboxymethyllysine 134. Since there are two Lys-134 residues per dimer, the second one must remain unmodified during the inhibition reaction and therefore may not be located near the D-ring of the steroid. This suggests that the steroid-binding site is constructed through an association of the two subunits in an AB to BA "sandwich" configuration. This interpretation lends support to the hypothesis placing the steroid at the interface between the subunits (Petra et al., 1983). This molecular arrangement would provide an explanation for the problem of a homodimeric protein molecule recognizing an asymmetric ligand. The AB/BA configuration would present two different surfaces to the two faces of the steroid. The results presented here support the postulation of the Lys-134 of one subunit on one surface near the D-ring, while the Lys-134 of the other subunit could be either at the opposite end of the steroid or well away from the steroid-binding site.

Analysis of SDS gels reveals that in addition to the $M_r = 44,000$ labeled SBP band there is a second radioactive band at about 28,000 (Fig. 2). The origin of this band remains unclear. It does not represent a contaminating protein because it is absent from the SDS-PAGE pattern of the unreacted protein (Fig. 2, lane 2). It appears that labeling at Lys-134 may promote a side reaction involving cleavage of the SBP polypeptide chain. In any case, this side reaction does not affect our interpretation of the data since the digested material from which the labeled peptides were isolated contained both $M_r = 44,000$ and 28,000 components and all the radioactive peptides that we isolated contained the sequence encompassing Lys-134.

DHTBr has the characteristics of an ideal affinity label for steroid-binding proteins and points the way to the design of isomeric labels with the reactive group at different positions. Placing the radioactive isotope in an acetoxy side chain is particularly advantageous because it provides an experimental means for removing the steroid while leaving the radioactive tag covalently attached to an amino acid residue. Removal of the hydrophobic steroid facilitates isolation of steroid-binding site peptides in good yield. The results presented here also show that the conditions used for removing the steroid moiety are mild enough to preserve the primary structure of the protein.

Although the nature of the data does not allow us to describe a specific role for Lys-134, its proximity to the 17β-OH of the steroid nucleus nevertheless suggests participation in the binding process through two possible mechanisms. First, the ε-amino group of Lys-134 could form a hydrogen bond with the 17β-OH group of the steroid either directly or indirectly. A recent report suggests that Met-139 is modified by the photoaffinity label, Δα-testosterone, which contains its reactive group at the 3β-position of the steroid (Grenot et al., 1988). It is therefore conceivable that the portion of the polypeptide chain encompassing these 2 residues (—PLTSKHPIMRIAL—) may have significant contact with a region of the steroid between the D-ring and the A-ring and may thus play an important role in the construction of the steroid-binding site. We have previously hypothesized that the alternating leucine segment located between residues 247 and 291 might represent part of the steroid binding site due to its hydrophobicity (Petra et al., 1988). Although we do not rule out that possibility, the present results indicate direct involvement of a different part of the protein.

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spectrometry measurements, Dr. Dave McCrae for his advice in the synthesis of steroid affinity labels, and Dr. Harry Charbonneau for valuable discussions during the course of this work.

Note Added in Proof—A paper was recently published suggesting that DHTBr alkylates His-235 instead of Lys-134 in human SBP (Khan and Rosner, 1990). Since a N'-carboxymethyllysine standard was not included in their analyses of alkylated protein hydrolysates, alklylation of a lysine residue cannot be ruled out in that work. Furthermore, Edman degradation of their radioactive peptide preparation yields phenylthiohydantoin-histidine at position 235 in relatively good yield for a charged amino acid indicating that His-235 is not alkylated.

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