Identification of Histidine and Methionine Residues in the Active Site of the Human Uterine Progesterone Receptor with the Affinity Labels 11α- and 16α-(Bromoacetoxy)progesterone†

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ABSTRACT: The affinity labels 11α- and 16α-(bromo[2′-^{3}H]acetoxy)progesterone (BAP) react covalently with amino acids present in the progesterone binding site of the human uterine progesterone receptor. Hydrolysis of the affinity labeled receptor followed by separation and analysis of the amino acid products demonstrated the sites of affinity labeling. The 11α-BAP alkylates the 1-position of a histidine residue. The 16α-BAP alkylates the 3-position of histidine, and a methionine residue. Affinity labeling did not occur in the presence of excess progesterone, and under the optimum conditions for affinity labeling of the receptor, heat-denatured receptor, bovine serum albumin, and 20β-hydroxysteroid dehydrogenase were not affinity labeled. This is the first report of the identification of specific amino acid residues in the binding site of a steroid hormone receptor.

A human uterine progesterone receptor has been purified by a combination of ammonium sulfate fractionation and affinity chromatography and found to have a molecular weight of 45 000 (Holmes et al., 1981; Smith et al., 1981). This purified receptor is possibly a proteolytic degradation product of the intact receptor such as the type IV receptor described in the chick oviduct (Sherman et al., 1976; Vedeckis et al., 1980) with which it shares similar physical properties (Smith et al., 1981). The size of the protein, however, is not important for affinity labeling studies provided the hormone binding site has been shown to be intact (Holmes et al., 1981; Smith et al., 1981).

The feasibility of using affinity labeling to determine the topography of an enzyme's steroid binding site has been demonstrated by the elegant studies of Warren's group (Ganguly & Warren, 1971; Chin & Warren, 1972; Sweet et al., 1972; Arias et al., 1973; Strickler et al., 1975). These workers determined the structure of the progesterone binding site of the enzyme 20β-hydroxysteroid dehydrogenase by utilizing a number of progesterone and cortisone derivatives bearing alkylating functions throughout the steroid nucleus. A similar approach has been used to identify amino acids present in the active site of ribonuclease (Gundlach et al., 1959; Crestfield et al., 1963), estradiol 17β-dehydrogenase (Pons et al., 1973a,b, 1976), and transcortin (Khan & Rosner, 1977; Le Gaillard & Dautrevaux, 1977). Until now similar procedures have not been applied to steroid hormone receptors because of the difficulty involved in achieving high levels of purity of these molecules. It is essential to use highly purified proteins since the high reactivity of the bromoacetate moiety on the steroid affinity label has the potential to interact with nucleophilic groups on any protein. Through the use of a highly purified receptor (Smith et al., 1981) and careful selection of the reaction conditions, we have shown that progesterone derivatives containing bromoacetoxy side chains at the 21-, 16α-, and 11α-positions were able to bind to and displace progesterone bound to the receptor and react covalently with amino acid residues at the binding site (Holmes et al., 1981).

The subsequent hydrolysis of the receptor–steroid adducts and the identification of nucleophilic amino acid residues present in the binding site of the human uterine progesterone receptor adjacent to the 16α- and 11α-positions of the progesterone nucleus will be described in this report.

Experimental Procedures

Materials

[1,2,6,7-^{3}H]Progesterone (97 Ci/mmol) was obtained from New England Nuclear Corp., Boston, MA. Bromo[2-^{3}H]-acetic acid (2.7 Ci/mmol) was purchased from Amersham, England. 16α- and 11α-hydroxyprogesterones were from Steraloids, Inc., Wilton, NH. Progesterone and glycolic acid were obtained from Sigma, St. Louis, MO. Chloroacetic acid was purchased from Fisher Scientific, Pittsburgh, PA.

Methods

Preparation of Radioinert 11α-(Bromoacetoxy)progesterone.[1] The procedure is similar to that described by Sweet & Warren (1972). 11α-Hydroxyprogesterone (1 g, 3 mmol) in dry methylene chloride (20 mL) was added to a solution containing bromoacetic acid (840 mg, 6 mmol) and dicyclohexylcarbodiimide (1.24 g, 6 mmol) in methylene chloride (20 mL) with stirring at 0 °C in an atmosphere of nitrogen. Pyridine (0.5 mL) was added, and stirring was continued at room temperature for 2 h. The mixture was then chilled in an ice bath and acetic acid (1 mL) added. The reaction mixture was allowed to remain chilled overnight, and then the precipitated dicyclohexylurea was removed by filtration. The filter cake was then washed with methylene chloride. The combined filtrates were evaporated to dryness under nitrogen. Acetone (50 mL) was added, and any undissolved residue was removed by filtration. The filtrate was cooled in ice, and water (200 mL) was added to precipitate crude 11α-(bromoacetoxy)progesterone, mp 122–130 °C. Recrystallization from ethyl acetate/petroleum ether furnished white crystals, mp 150–152 °C, lit. mp 152–154 °C (Sweet & Warren, 1972).

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[1] Abbreviations: 11α-(bromoacetoxy)progesterone, 11α-(bromoacetoxy)-4-pregnene-3,20-dione; 16α-(bromoacetoxy)progesterone, 16α-(bromoacetoxy)-4-pregnene-3,20-dione; $NaDodSO_4$, sodium dodecyl sulfate; CM, carboxymethyl; BAP, (bromoacetoxy)progesterone; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-1729$01.50/0

Preparation of 11α-(Bromo[2′-^{3}H]acetoxy)progesterone (2.7 Ci/mmol). A methylene chloride solution of 11α-hydroxyprogesterone (52 μg, 1.86 μmol) was added to a microvial equipped with a Teflon spin vane and septum. Bromo[2-^{3}H]acetic acid (1.86 μmol) was added in dry freshly distilled methylene chloride (450 μL). The mixture was stirred under an atmosphere of nitrogen in an ice bath, and dicyclohexylcarbodiimide (421 μg, 2.04 μmol) was added in dry methylene chloride (19.5 μL). After 5 min dry pyridine (1 μL) was added, and stirring was continued for 1 h at 0 °C and 1 h at 22 °C. The entire solution was then applied to a silica gel TLC plate and the 11α-(bromo[2′-^{3}H]acetoxy)progesterone was purified by chromatography by using a ratio of 30/70 ethyl acetate/petroleum ether. The band of silica gel containing the pure product was scraped from the TLC plate and then transferred to a Pasteur pipet for elution of the steroid from the silica gel with methylene chloride (15 × 1 mL). The methylene chloride was then evaporated, and the residue was rechromatographed to furnish pure 11α-(bromo[2′-^{3}H]acetoxy)progesterone in 20% yield with a specific activity of 2.7 Ci/mmol.

16α-(Bromoacetoxy)progesterone. The radiolabeled 16α-(bromo[2′-^{3}H]acetoxy)progesterone (2.0 Ci/mmol) was a gift from Dr. Gary Murdoch, Department of Ob/Gyn, Washington University of Medicine, St. Louis, MO, and the radioinert compound was prepared according to Sweet et al. (1972).

Purification of the Human Uterine Progesterone Receptor and Affinity Labeling. Details of the methodology have been described before (Holmes et al., 1981; Smith et al., 1981). Briefly, fresh uterine specimens were diced, minced with a meat grinder, and then homogenized with 4 volumes (w/v) of TETG buffer (10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 12 mM monothioglycerol, and 10% v/v glycerol) in a Polytron (Brinkmann) for three 5-s pulses. All procedures were performed at 4 °C unless otherwise stated. The resulting homogenate was centrifuged at 10000*g* for 10 min and the supernatant further centrifuged for 1 h at 105000*g*. Saturated ammonium sulfate was added to the resulting cytosol until a final concentration of 30% was obtained. After centrifugation (10000*g* for 10 min) the pellet was redissolved in TETG buffer and centrifuged at 200000*g* for 20 min to sediment insoluble material. The supernatant was then incubated with affinity resin (Smith et al., 1981) for 18 h at 4 °C. The resin was separated by centrifugation, washed with 0.5 M NaSCN, TETG buffer, 50% glycerol in TETG for 30 min, and finally with TETG buffer. The bound receptor was eluted from the resin by incubation with 10 μM progesterone containing 100 μCi of [^{3}H]progesterone in TETG buffer for 30 min at 22 °C. The receptor–progesterone complex was then isolated by chromatography on Sephadex G-75 (equilibrated with 10 mM phosphate buffer, pH 7.0). To the excluded fractions containing the receptor was added 50–500 nM radiolabeled 16α- or 11α-(bromoacetoxy)progesterone, and the solution was incubated for 30 min at 4 °C and then 1–4 h at 22 °C with gentle mixing (rotating the tube at 1 rpm). The resulting affinity labeled receptor was again isolated by chromatography on Sephadex G-75.

Preparation of Carboxymethylated Amino Acid Standards. The carboxymethylsulfonium salt of methionine was prepared according to Gundlach et al. (1959). *S*-Methylthioglycolic acid (Goren et al., 1968) and *S*-(carboxymethyl)homocysteine (Armstrong & Lewis, 1951) were prepared by established procedures. A mixture of (dicarboxymethyl)histidine and 1- and 3-(carboxymethyl)histidine was prepared as described by Crestfield et al. (1963). However, adequate separation of the three histidine derivatives by the previously published method proved unsuccessful. Separation was accomplished by applying 0.5 mL (50 mg) of histidine derivatives to a column (0.9 × 55 cm) of Beckman PA-28 resin, using a flow rate of 1.0 mL/min, PSI 500, and 0.2 M citrate buffer, pH 3.25, to 195 min and then 0.2 M citrate buffer, pH 4.25. Temperature was 30 °C for 115 min and then 50 °C.

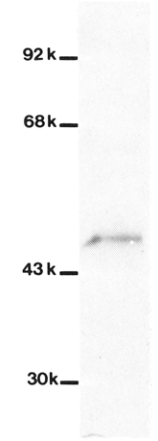


FIGURE 1: NaDodSO$_4$/polyacrylamide gel electrophoresis of the human progesterone receptor.

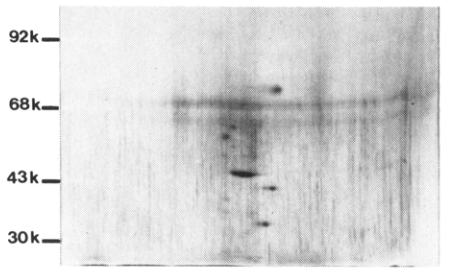


FIGURE 2: Two-dimensional gel electrophoresis of the human progesterone receptor.

Gel Electrophoresis. Sodium dodecyl sulfate (NaDodSO$_4$)/polyacrylamide slab gel electrophoresis was performed as described by Laemmli (1970) with a 12.5% gel. Two-dimensional gel electrophoresis was performed by the method of O'Farrell (1975) on a 4.5% acrylamide tube gel with pH 3.5–10 ampholites (LKB, Rockville, MD) for the first dimension and a 10% acrylamide gel for the second dimension. The gel was silver stained according to the method of Wray et al. (1981).

Results

Purity of the Receptor Preparation. The human progesterone receptor when subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis with Coomassie blue staining was approximately 95% pure and had a molecular weight of 45 000 (Figure 1; Holmes et al., 1981; Smith et al., 1981). However, silver staining (which is not quantitative) demonstrated the presence of five minor proteins within the preparation (Figure 2). Two-dimensional gel electrophoresis revealed a broad band at a molecular weight of 46 000 and p*I* of 6.5.

Evidence That 16α-BAP and 11α-BAP Bind to the Progesterone-Specific Binding Site of the Receptor. We have previously shown that by applying the receptor labeled with either 11α- or 16α-[2′-^{3}H]BAP to NaDodSO$_4$/polyacrylamide gels that (1) only the M_r 45 000 band was radioactively labeled and (2) the binding was specific as 50 nM 11α- or 16α-[2′-^{3}H]BAP in the presence of 5 μM progesterone did not affinity label the receptor. We have also demonstrated that the above

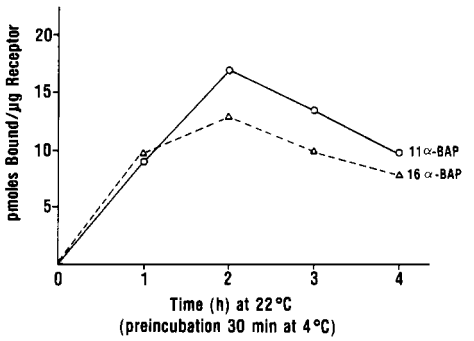


FIGURE 3: Time course of affinity labeling the receptor with 16α- and 11α-(bromo[2′-^{3}H]acetoxy)progesterone (BAP). The receptor was incubated with 100 nM affinity label for 30 min at 4 °C and 1–4 h at 22 °C and then isolated by chromatography on Sephadex G-75.

affinity labels can bind to and displace progesterone bound to the receptor (Holmes et al., 1981).

To further confirm that the high affinity binding was to progesterone receptor rather than some other protein, 100 nM 16α-[2′-^{3}H]BAP was incubated with 1 μg of bovine serum albumin, heat-inactivated receptor, or 20β-hydroxysteroid dehydrogenase for 30 min at 4 °C and then 2 h at 22 °C. The proteins were then isolated by chromatography on Sephadex G-75. One microgram of protein was selected since it was typical of the amount of receptor used in our receptor affinity labeling experiments. The steroid dehydrogenase was used since it had already been the study of affinity labeling; its K_m for 16α-BAP is 1.5×10^{-4} M (Sweet et al., 1972). The albumin and heat-inactivated receptor showed no affinity labeling while the enzyme had 0.2 pmol of BAP bound/μg of protein. When the concentration of 16α-BAP was increased to 1 μM, 21.2 pmol was bound per μg of enzyme (1 μg of enzyme = 10 pmol). In contrast, 17.0 pmol of BAP was bound per μg of protein when the intact receptor and 100 nM 16α-BAP were used. The theoretical amount of BAP bound assuming a single binding site per receptor molecule is 23 pmol/μg of protein.

Time Course of Affinity Labeling with 16α- and 11α-[2′-^{3}H]BAP. Both affinity labels showed a similar effect in that the peak number of counts incorporated into the receptor was achieved after an incubation of 30 min at 4 °C plus 2 h at 22 °C (Figure 3). The two affinity labels were incubated with receptor for up to 4 h at 22 °C. Longer periods of incubation result in denaturation of the receptor (Holmes et al., 1981). This fact is evident from the decreased amount of steroid bound to the receptor after a 4-h incubation which represents a loss of loosely bound steroid.

Estimation of the Nonspecific Binding of the Affinity Labels to the Receptor. The bromoacetoxy group on the affinity labels is very reactive, and the amount of nonspecific binding (NSB) of this moiety to nucleophilic amino acid residues other than those in the binding site of the receptor must be estimated. If the receptor is incubated with excess unlabeled progesterone (1 μM) before adding the ^{3}H affinity label, the only ^{3}H binding occurring other than that due to exchange will be nonspecific (Table I). The lowest apparent nonspecific binding expressed as a percentage of the total occurred with 100 nM affinity label. Even at concentrations of BAP of 500 nM the NSB was only 5–6%.

The receptor was heated with $NaDodSO_4$ to remove any noncovalently attached steroid molecules. Approximately 30–40% of the counts associated with the receptor after affinity labeling are lost with this treatment. For comparison, when [^{3}H]progesterone was substituted for the affinity label, 99% of the counts were released from the receptor.

Table I: Nonspecific Binding (NSB) of the Affinity Labels[a]

	progesterone	pmol/μg of receptor	% NSB
11α-[2′-^{3}H]BAP			
50 nM	−	3.26	
50 nM	+	0.28	8.6
100 nM	−	12.80	
100 nM	+	0.44	3.4
500 nM	−	14.64	
500 nM	+	0.72	4.9
16α-[2′-^{3}H]BAP			
500 nM	−	16.30	
500 nM	+	0.98	6.0
bromoacetic acid			
500 nM	+	0.64	

[a] The receptor was incubated with or without progesterone (1 μM) plus 50, 100, or 500 nM of 11α-(bromo[2′-^{3}H]acetoxy)-progesterone (BAP), 500 nM bromo[^{3}H]acetic acid, or 500 nM 16α-[2′-^{3}H]BAP for 30 min at 4 °C and 2 h at 22 °C, isolated by chromatography on Sephadex G-75, lyophilized, heated for 30 min at 85 °C with 1% $NaDodSO_4$ solution, and again isolated by G-75.

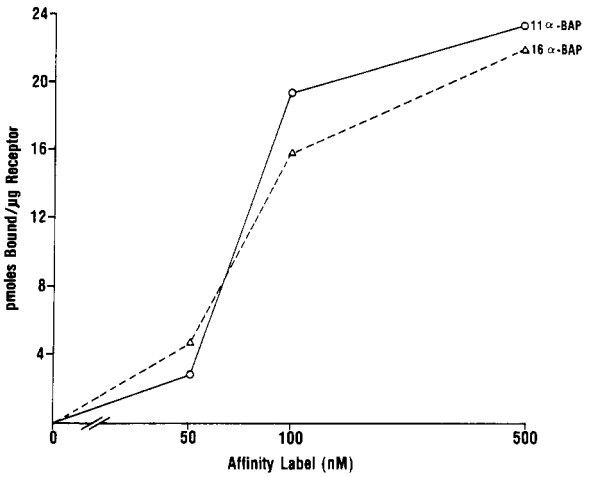
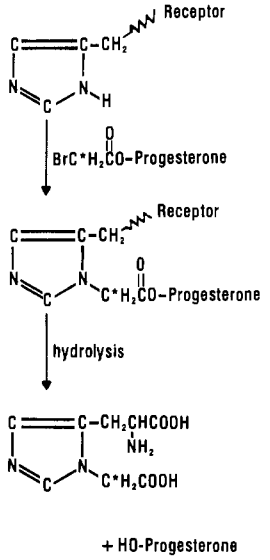


FIGURE 4: Effect of increasing concentration of 16α- and 11α-(bromo[2′-^{3}H]acetoxy)progesterone (BAP) on affinity labeling the receptor. The receptor was incubated with 50–500 nM affinity label for 30 min at 4 °C plus 2 h at 22 °C and then isolated by chromatography on Sephadex G-75.

Figure 4 shows that the dose–response curves begin to plateau with 100 and 500 nM concentrations of affinity label. However, as the nonspecific binding using 100 nM of the 11α-BAP was lower than 500 nM (Table I), 100 nM was the concentration selected to study affinity labeling of the receptor.

Preparation and Isolation of Standards To Identify the Hydrolysis Products of the Affinity Labeled Receptor. A mixture of the (carboxymethyl)histidines was prepared as described under Methods. The mixture was fractionated on a Beckman PA-28 resin. An aliquot of each fraction was reacted with ninhydrin, and five ninhydrin-positive peaks were detected: (A) 10–15 mL, (B) 75–85 mL, (C) 105–125 mL, (D) 130–150 mL, and (E) 235–250 mL. Fraction A eluted at the position of urea, fraction E was histidine, and fractions B, C, and D corresponded to di-CM-histidine, 1-CM-histidine, and 3-CM-histidine, respectively (Crestfield et al., 1963). Fractions B, C, and D were then desalted on Dowex 50-X8 H^+ form, lyophilized, and suspended in glass-distilled H_2O. Amino acid analysis (Beckman Model 121 modified for Dionex 4A resin) demonstrated that fraction B was 95% pure 1,3-di-CM-histidine, C was 80% pure 1-CM-histidine (9% di-CM-histidine, 11% 3-CM-histidine), and D was 96% pure 3-CM-histidine (2% di-CM-histidine, 2% 1-CM-histidine).

Scheme I: Example of the Interaction of 11α-[2′-^{3}H]BAP with a Histidine Residue at the Progesterone Receptor Binding Site, Resulting in the Formation of 1-(Carboxymethyl)histidine



The CM derivatives of cysteine and methionine were either purchased or obtained by published procedures as described under Experimental Procedures.

Separation of the Carboxymethylated Amino Acids. Under the conditions of affinity labeling (10 mM phosphate buffer, pH 7.0), it has been shown that the only amino acid residues which react with the BAPs are histidine, methionine, and cysteine (Sweet et al., 1972; Arias et al., 1973). Alkylation of these nucleophilic residues by the [^{3}H]BAP derivatives occurs by S_N2 displacement of the bromine atom resulting in covalent linkage of the ^{3}H-labeled steroid to the particular amino acid residue adjacent to the progesterone binding site. Scheme I is an example of how 11α-[2′-^{3}H]BAP can interact with a histidine residue. Hydrolysis of this progesterone–receptor adduct will furnish a ^{3}H-labeled carboxymethyl amino acid. In addition to the example depicted in Scheme I it is also possible for histidine to react with the BAP at N-3 of the imidazole ring.

Interaction of [2′-^{3}H]BAP with a methionine residue and subsequent hydrolysis furnished the CM-sulfonium salt of methionine. Under the conditions used for the hydrolysis of the receptor–BAP adduct the sulfonium salt decomposed into seven different products (Goren et al., 1968). However, of these only glycolic acid, chloroacetic acid, CM-homocysteine, and *S*-methylthioglycolic acid retain the ^{3}H atom; thus, only these four decomposition products are relevant to the affinity labeling studies.

Separation of the possible products of hydrolysis of the affinity labeled receptor, 1- and 3-CM-histidines, 1,3-di-CM-histidine, glycolic acid, CM-homocysteine, *S*-methylthioglycolic acid, chloroacetic acid, and CM-cysteine, was accomplished with a high-pressure liquid chromatograph (HPLC). This instrument utilized a Model 6000A delivery system, a Model 46K valve injector fitted with a 2-mL injection loop, and two 0.4 × 30 cm, stainless steel columns packed with μBondapak NH_2 (Waters Associates). The above standards were eluted from the columns with 15% CH_3CN/85% 0.2 M acetate buffer, pH 3.25, at a flow rate of 2.0 mL/min. Detection was accomplished with a differential refractometer, R401 (Waters Associates). The retention time of each of these components in their order of elution is given in Table II.

Identification of Amino Acids in the Receptor Adjacent to the 11α- and 16α-Positions of the Progesterone Molecule. The

Table II: Retention Times of Standards[a]

standard	retention time (min)
1-CM-histidine	7.2
glycolic acid	8.6
CM-homocysteine	10.8
CM-cysteine	14.4
S-methylthioglycolic acid	16.2
chloroacetic acid	18.0
3-CM-histidine	21.4
di-CM-histidine	23.6

[a] Mobile phase, 15% CH_3CN/0.2 M acetate buffer, pH 3.25; flow rate 2.0 mL/min; two μBondapak NH_2 columns (30 × 0.4 cm); sample, 250–500 μg of each standard in 150 μL of 0.2 M acetate buffer, pH 3.25.

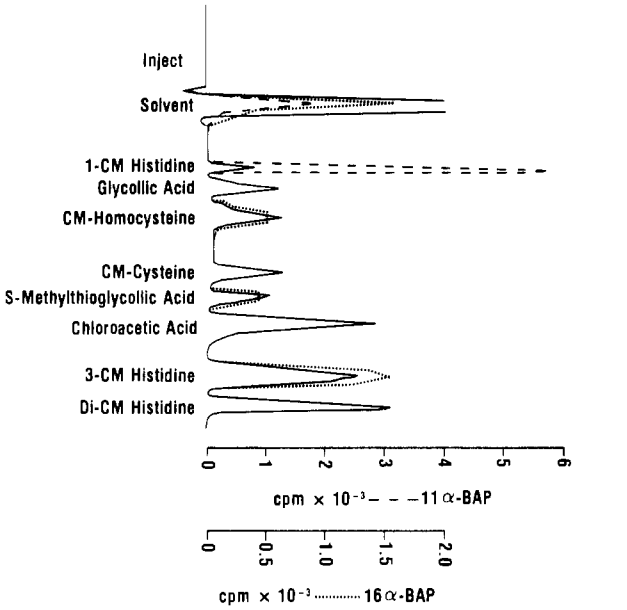


FIGURE 5: Radioactivity incorporated into each standard on affinity labeling the receptor with 100 nM 16α- and 11α-(bromo[2′-^{3}H]-acetoxy)progesterone (BAP). With 11α-BAP 17 979 cpm was present in the 1-CM-histidine peak and 3319 cpm in the solvent peak. For 16α-BAP the figures were 5272 cpm for the 3-CM-histidine peak, 2052 cpm for the methionine standards, and 3576 cpm in the solvent peak.

affinity labeled receptor was lyophilized, hydrolyzed in vacuo at 110 °C in 6 M HCl for 24 h, and then concentrated by rotary evaporation or lyophilization. To the lyophilysate was added 250–500 μg of each standard in 150 μL of 0.2 M acetate buffer, pH 3.25. The eluant from the HPLC columns was collected in 0.5-mL fractions directly from the refractometer and 5 mL of Scintiverse (Fisher) added. The amount of radioactivity in each fraction was then correlated to the retention time of each standard. When the HCl hydrolysate of the receptor affinity labeled with 11α-[2′-^{3}H]BAP was applied to the HPLC, the elution pattern showed one radioactive peak containing 17 979 cpm corresponding to 1-CM-histidine (Figure 5). There were 3319 cpm present in the solvent peak, indicative of tritium exchange. With 16α-BAP (Figure 5) three radioactive peaks were observed, 3-CM-histidine (5272 cpm), CM-homocysteine, and *S*-methylthioglycolic acid (2052 cpm), indicating that a histidine residue and a methionine residue were labeled in the ratio 5:2, respectively. Only approximately 5% of the counts associated with the receptor after affinity labeling with 11α-BAP were lost during posthydrolysis lyophilization. However, 20–30% of the counts were lost after lyophilization when the receptor was affinity labeled with 16α-BAP. It is possible that a cysteine, glutamate, or aspartate

residue could also have been affinity labeled and subsequently not detected due to tritium exchange or hydrolysis. However, since the decomposition products of the CM-sulfonium salt of methionine are also volatile and we have demonstrated the presence of a methionine residue, it is most likely that these losses are associated with decomposition of the [^{3}H]CM-sulfonium salt. Affinity labeling of the receptor was carried out at least 3 times with each derivative and gave identical results as to which amino acid residue was labeled. The only inconsistency in the results was with the relative ratio of the four decomposition products of the CM-sulfonium salt of methionine. However, a similar observation was made when a methionine residue was found in the active site of the enzyme 20β-hydroxysteroid dehydrogenase (Strickler et al., 1975).

Discussion

We have shown that 11α-[2′-^{3}H]BAP and 16α-[2′-^{3}H]BAP covalently bind to the highly purified human progesterone receptor and that this reaction is blocked by the presence of excess radioinert progesterone. Furthermore, no covalent binding is observed when the reaction is attempted with bromo[^{3}H]acetic acid or [^{3}H]progesterone (Holmes et al., 1981; this report). We have optimized the conditions for affinity labeling of the receptor with respect to concentration of BAP and time. By use of these conditions the affinity labeling of heat-denatured receptor, bovine serum albumin, and 20β-hydroxysteroid dehydrogenase was attempted. The only protein of these three to be labeled was the enzyme, but the affinity labeling was very inefficient. However, when the concentrations of BAP were increased to 1 μM, 21.2 pmol of BAP was incorporated per pmol of enzyme, in agreement with the observations of Sweet et al. (1972). The results show that the affinity labeling of the receptor described herein is both specific for the progesterone binding site and specific for the progesterone receptor protein.

It was necessary to obtain the expected hydrolysis products of the receptor–steroid adducts to be used as standards in the subsequent analyses. We encountered considerable difficulty in the chromatographic separation of 1-CM-histidine, 3-CM-histidine and the 1,3-di-CM-histidine when we used published procedures. We therefore developed an improved method using a Beckman P-28 ion-exchange resin to purify these standards. Separation and analysis of all of the expected hydrolysis products, CM-cysteine, the three CM-histidines, and the decomposition products of CM-sulfonium salt of methionine, were accomplished by HPLC with a reverse phase ion-exchange resin and a refractive index detector. This new procedure was developed because decomposition products of CM-sulfonium salt of methionine do not give the ninhydrin reaction and would therefore not be detectable on a conventional amino acid analyzer.

The particular amino acid residues alkylated by the 11α- and 16α-BAPs have been identified by hydrolysis of the steroid–receptor adducts followed by separation of the resulting carboxymethylated amino acids using HPLC. These amino acids were identified as histidine reacting at its 1-position with the 11α derivative, the 16α-BAP reacting with a histidine at the 3-position and with methionine. Although the ratio of histidine to methionine alkylated by 16α-BAP was found to be 5:2, this ratio is perhaps closer to 1:1 because of the volatility of the decomposition products of CM-sulfonium salt of methionine. Indeed, previous studies have shown that in the freeze-drying of the hydrolysis products, prior to analysis, only 70% of the decomposition products of CM-sulfonium salt of methionine are apparently recovered (Le Gaillard & Dautrevaux, 1977). The fact that two amino acid residues are labeled by the 16α-BAP is explained by the fact that, although the progesterone binding site itself imposes strict limits on the flexibility of the progesterone molecule, no similar restriction need be placed on the bromoacetoxy group which can rotate 360° at approximately 5 Å from the steroid nucleus itself. Thus any histidine, methionine, or cysteine residue located in this region would predictably be alkylated.

There is evidence to suggest that progesterone binds to the receptor by at least two hydrogen bonds to oxygen atoms at the 3- and 20-positions of the steroid molecule (Deletre et al., 1980). Our data indicate that a methionine or histidine residue adjacent to the D ring may act as a hydrogen bond donor to the oxygen atom at the 20-position of the steroid molecule (O-20). Examination of steric models shows that (1) the 11α- and 16α-alkylating groups could be reacting with the same histidine (horizontal to the α-plane of the C and D rings; however, this histidine could not act as hydrogen bond donor to the O-20) and (2) the 11α- and 16α-BAPs may be reacting with separate histidines, either of which (or both) could be hydrogen bond donors to the O-20. With respect to the methionine residue in the vicinity of the 16α-position, enhanced binding of 21-fluoroprogesterone with the human receptor has been reported (Smith et al., 1974). Furthermore, sulfhydryl blocking agents decrease progesterone binding (Sherman et al., 1970). It has been proposed that a sulfhydryl group could interact with the carbonyl group at C-20 to form an α-hydroxy sulfide. The methionine residue may also react in this matter.

Steroid hormones appear to enter the corticosteroid binding globulin with the A ring pointed to the interior (Basset et al., 1975). An inverted A ring structure has been proposed for high affinity binding of progesterone analogues to the receptor in rabbit uterus (Duax et al., 1978a). There appears to be a loose fit between the steroid and receptor on the α- and β-faces of the B, C, and D rings (Duax et al., 1978b). Although the A ring appears to predominate in binding, an interesting comparison can be made between the human progesterone receptor and human transcortin which has also been subjected to affinity labeling by using progesterone analogues with bromoacetoxy groups on the C and D rings. With transcortin, a methionine residue was located near the 11α-position, and histidine and methionine residues were located adjacent to the 16α- and 17α-positions. Our results with the receptor concerning the binding site around the D ring of progesterone are similar in that histidine and methionine residues are present. However, at the C ring the receptor differs from transcortin in that histidine was present near the 11α-position. The affinity of progesterone and cortisol for transcortin is very similar (K_d = 10^{-8}–10^{-9} M) whereas the relative binding affinity of progesterone for the receptor (3 × 10^{-9} M) is approximately 1000 times greater than for cortisol (Smith et al., 1974). Since the 11β-hydroxyl group does not influence the shape of the A ring to any great extent (Duax & Norton, 1975), the histidine adjacent to the 11α-position may play an important role in the specificity of the receptor for certain steroids.

We are continuing to map the hormone binding site of the human uterine progesterone receptor with the 2α-BAP, 6β-BAP, and 21-BAP affinity labels. Using this methodological approach we should be able to design in a logical manner more specific agonists and antagonists of progesterone.

Acknowledgments

We express our appreciation to Dr. J. H. Brown for his advice and help with the separation of the (carboxymethyl)-histidines.

Registry No. 16α-BAP, 84623-69-8; progesterone, 57-83-0; histidine, 71-00-1; methionine, 63-68-3; 11α-hydroxyprogesterone, 80-75-1; 11α-[2′-^{3}H]BAP, 84623-70-1; 11α-BAP, 36049-50-0.

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Spectral Studies on the Calcium Binding Properties of Bovine Brain S-100b Protein†

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ABSTRACT: The effect of Ca^{2+} binding on the circular dichroism (CD) and 270-MHz proton nuclear magnetic resonance (NMR) spectra of brain-specific S-100b calcium binding protein has been examined at two pH values, 8.5 and 7.5. At pH 8.5, S-100b protein binds two Ca^{2+} per monomer with K_d values of 6×10^{-5} and 2×10^{-4} M, whereas at pH 7.5, the protein binds only one Ca^{2+} per monomer with a K_d of 2×10^{-4} M. The presence of K^+ inhibits the binding of Ca^{2+} to the higher affinity site at pH 8.5, and the affinity for calcium is lowered to $K_d = 8.5 \times 10^{-4}$ M. Mg^{2+} has no effect on protein conformation. In the absence of Ca^{2+}, S-100b undergoes a conformational change when the protein is titrated from pH 8.6 to 6.0. Addition of Ca^{2+} perturbed the environment of tyrosine and phenylalanine residues as measured by ultraviolet difference spectroscopy and ^{1}H NMR. CD melt experiments and far-ultraviolet CD studies at alkaline pH and NMR experiments suggest that the protein is more stable in the presence of Ca^{2+}. The single tyrosine residue in the protein ionizes only after the protein is denatured by exposure to high pH.

The characterization of proteins specific to nervous tissue is essential for studying the structure and the function of the nervous system at the molecular level. The brain-specific S-100 protein is found primarily in glial cells (Moore, 1965) and represents up to 0.2% of the total soluble brain protein. The biological function of this protein is unknown; however, previous results suggest a role for it in the function or development of the nervous system (Hyden & Lange, 1970; Calissano & Bangham, 1971; Calissano et al., 1974). S-100 protein, which is a water-soluble, highly acidic calcium binding protein (Calissano et al., 1976), is actually a mixture of two components, S-100a and S-100b, with a subunit composition of $\alpha\beta$ and β_2, respectively (Isobe & Okuyama, 1981). S-100b exists as a dimer of 21 000 molecular weight in native solvents,

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‡ J.G.S. was supported by a studentship from the Alberta Heritage Foundation for Medical Research.

0006-2960/83/0422-1734$01.50/0