Affinity Labeling of the Androgen Receptor in Rat Prostate Cytosol with 17β-[(Bromoacetyl)oxy]-5α-androstan-3-one†

Ching H. Chang,‡ Thomas J. Lobl, David R. Rowley,§ and Donald J. Tindall*,⊥

ABSTRACT: An androgen affinity label, 17β-[(bromoacetyl)-oxy]-5α-androstan-3-one, has been synthesized in both radioactive and nonradioactive forms. The affinity label (170 Ci/mmol) was characterized and found to have a high degree of purity. Affinity labeling of the androgen receptor from rat ventral prostate was androgen specific and appeared to be directed at the steroid binding site of the protein. Covalent binding was achieved at 0 °C; however, heat treatment at 23 °C for 30 min enhanced covalent binding by 31%. The covalently bound steroid was resistant to extraction with organic solvents and precipitation with trichloroacetate. The Stokes radius (4.2 nm) and sedimentation coefficient (4.5 S) were identical with those found for receptor bound noncovalently to dihydrotestosterone. Gel electrophoresis of the affinity-labeled receptor under denaturing conditions revealed a molecular weight of 86 000. The same molecular weight was observed for the receptor from rat seminal vesicle. This affinity label will be useful in future studies on the structure and function of androgen receptors.

Electrophilic steroid affinity labels have been used extensively to characterize a number of steroid-metabolizing enzymes (Ganguly & Warren, 1971; Sweet et al., 1972; Strickler et al., 1975; Sweet & Samant, 1980; Thomas & Strickler, 1983) and more recently steroid receptor proteins (Simons et al., 1983; Katzenellenbogen et al., 1983; Weisz et al., 1983; Holmes & Smith, 1983). These compounds usually possess a good "leaving" group such as a bromine atom or a methylsulfonyloxy (mesylate) moiety, which is displaced, thus allowing the steroid to bind covalently to the active site of the protein.

Our laboratory has been interested in characterizing the physicochemical and steroid binding properties of androgen receptors from several androgen responsive tissues (Chang et al., 1982, 1983; Chang & Tindall, 1983). To this end we have purified and characterized the androgen receptors from steer seminal vesicle (Chang et al., 1982) and rat ventral prostate (Chang et al., 1983). Preliminary results suggested that we could use an electrophilic affinity label, dihydrotestosterone 17β-bromoacetate,[1] to covalently tag this receptor protein and identify its molecular weight by polyacrylamide gel electrophoresis under denaturing conditions. In order to more fully define the properties of this affinity label, we have further studied the conditions for this reaction. This paper describes the synthesis of dihydrotestosterone 17β-bromoacetate and its characterization, evidence that this compound binds to the active site of the receptor protein and proof that the steroid–receptor complex is joined by a covalent bond.

Materials and Methods

The following materials were obtained: [1,2,4,5,6,7-$^3H_6$]-dihydrotestosterone (143 Ci/mmol) from Amersham; [1,2,4,5,6,7,16,17-$^3H_8$]dihydrotestosterone (170 Ci/mmol) and Enhance from New England Nuclear (other steroids were from Steraloids); ammonium sulfate and Tris (base) from Schwarz/Mann; deoxyribonucleic acid (calf thymus), bovine liver catalase, bovine γ-globulin, myoglobin, bovine serum albumin, and dithiothreitol from Sigma; activated charcoal (Norit A) from J. T. Baker; Gelatin from Knox-gel; Na_2-EDTA, glycerol, and sodium chloride from Fisher; *N,N'*-methylenebis(acrylamide), acrylamide, and *N,N,N',N'*-tetramethylethylenediamine from Eastman; X-oMat AR film from Eastman Kodak Co.; blue dextran, dextran T-70, and CNBr-activated Sepharose 4B from Pharmacia; agarose A-0.5m from Bio-Rad. Leupeptin was a gift from the United States–Japan Cooperative Cancer Research Program.

17β-[(Bromoacetyl)oxy]-5α-androstan-3-one. A solution of bromoacetyl bromide (3.8 g, 18.9 mmol) in 25 mL of toluene was added dropwise over 1 h to a solution of dihydrotestosterone (5 g, 17.2 mmol; The Upjohn Co.) in 50 mL of toluene cooled in an ice bath. This was followed by the dropwise addition of diisopropylethylamine (3.4 mL, 10.5 mmol). After 8 h at room temperature the mixture was filtered. The mother liquor was extracted with 5% sodium bicarbonate and H_2O and dried with Na_2SO_4, and the solvent was removed in vacuo. This produced 7.5 g of residue. The residue was chromatographed on silica gel eluting with a linear gradient of hexane/ethyl acetate, 8:2 to 6:4. The desired material was crystallized from acetone/hexane to give analytically pure material (4.6 g, 11.2 mmol, 65%): mp 132–133 °C; NMR ($CDCl_3$) δ 0.84 (s, 18-CH_3), 1.01 (s, 19-CH_3), 3.82 (s, CH_2-Br), and 4.66 (t, J = 8 Hz, 17α-H); mass spectrum, *m/e* (relative intensity) 410, 412 (P^+), 272; IR (Nujol) 1745 (CO–O), 1716 (3-CO), 1464, 1442, 1288, 1190, 1124 cm^{-1}; UV, only end absorption. Anal. Calcd for $C_{21}H_{31}BrO_3$: C, 61.31; H, 7.60; Br, 19.42. Found: C, 61.03; H, 7.67; Br, 19.56. The chemical structure of 17β-[(bromoacetyl)oxy]-5α-androstan-3-one is shown in Figure 1 (see Appendix).

17β-[(Bromoacetyl)oxy][1,2,4,5,6,7,16,17-$^3H_8$]-5α-androstan-3-one. [1,2,4,5,6,7,16,17-$^3H_8$]Dihydrotestosterone

† From the Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030 (C.H.C., D.R.R., and D.J.T.), and Fertility Research, The Upjohn Company, Kalamazoo, Michigan 49001 (T.J.L.). *Received October 18, 1983.* This research was supported in part by Grants HD-12788 and CA-32387 from the National Institutes of Health.

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[1] Abbreviations: dihydrotestosterone 17β-bromoacetate, 17β-[(bromoacetyl)oxy]-5α-androstan-3-one; dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; leupeptin, a mixture of *N*-acetyl- and *N*-propionyl-L-leucyl-L-leucyl-DL-arginine aldehyde hydrochlorides; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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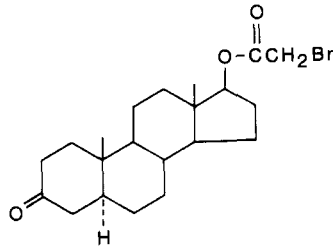

FIGURE 1: Chemical structure of 17β-[(bromoacetyl)oxy]-5α-androstan-3-one.

(1.9 μg, 4.5 nmol) was transferred to a test tube, and the solvent was evaporated under a stream of N_2. Fifty microliters each of stock solutions A and B was added respectively (stock A, 10 μL of bromoacetyl bromide in 10 mL of toluene, 2.3 ng/μL; stock B, 20 μL of diisopropylethylamine in 10 mL of toluene, 1.5 ng/μL). After the solution was incubated for 4.5 h, the solvent was evaporated under a stream of N_2, and an additional 50 μL each of stock solutions A and B was added. After the solution was incubated for 18 h, the solvent was evaporated under a stream of N_2, and the residue was applied to a micro silica gel column (400 mg of absorbent, 70–230 mesh; hexane/ethyl acetate, 6:4; 0.5-mL fractions). Pure affinity label was found in fractions 2 and 3. A radiochromatogram of the thin-layer chromatography eluate showed only one peak of radioactive material (see Appendix).

Buffers. The following buffers were used: TED buffer (50 mM Tris-HCl buffer containing 1.5 mM EDTA and 1.5 mM dithiothreitol, pH 7.4 at 22 °C); TEDG buffer (TED buffer containing 20% glycerol); TEDN buffer (TED buffer containing 0.5 M NaCl); TEDGN buffer (TED buffer containing 10% glycerol and 0.5 M NaCl); TEDGL buffer (TEDG buffer containing 10 μg/mL leupeptin); PE buffer (5 mM phosphate buffer containing 0.1 mM EDTA, pH 7.0 at 22 °C).

Preparation and Labeling of Cytosol. Male Sprague-Dawley rats (3–4 months old) were sacrificed 24 h after orchiectomy. Ventral prostate lobes were removed and frozen quickly in liquid nitrogen prior to storage at −90 °C. Cytosol was prepared in TEDGL buffer as described previously (Chang et al., 1983). The cytosol was precipitated with 40% ammonium sulfate, resuspended in PE buffer, and incubated with radioactive steroid in the presence or absence of 100-fold excess unlabeled ligand.

In order to confirm the stability of the steroid [^{3}H]dihydrotestosterone 17β-bromoacetate, we examined the steroid on silica gel either after a 3-h incubation at 0 °C or after a 2.5-h incubation at 0 °C plus another 30 min at 23 °C. The results indicated that no conversion of the steroid to [^{3}H]dihydrotestosterone was detected under both experimental conditions.

Binding Assays. Androgen receptor activity was determined with a charcoal binding assay as described by Korenman (1969) with minor modifications described previously (Chang et al., 1982). In some experiments [^{3}H]dihydrotestosterone was exchanged for nonradioactive steroid bound to the receptor under exchange conditions: unlabeled androgens were removed by charcoal adsorption, and [^{3}H]dihydrotestosterone was exchanged onto the receptor by warming the preparations at 30 °C for 30 min. Bound radioactivity was then determined by the charcoal binding assay described above.

Molecular Weight Determinations. Gel filtration chromatography was carried out in an agarose A-0.5m column (1.8 × 50 cm) as described previously (Chang et al., 1982). Sucrose gradient sedimentation was performed on gradients (5 mL) containing 2–20% sucrose in TEDGN buffer as described previously (Chang et al., 1982). Stokes radii and sedimentation coefficients of standard proteins were used to determine molecular weights and frictional ratios as described by Siegel & Monty (1966).

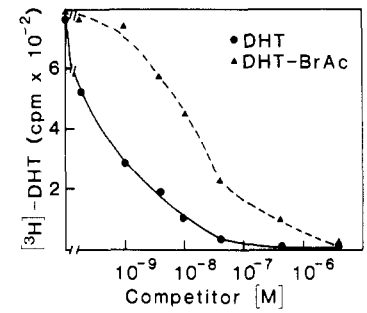

FIGURE 2: Competition of binding in rat prostate cytosol. Aliquots (250 μL) of prostate cytosol were incubated for 18 h at 0 °C with 1 nM [^{3}H]dihydrotestosterone in the absence or presence of different concentrations of either dihydrotestosterone or dihydrotestosterone 17β-bromoacetate. Bound activity was determined by the charcoal binding assay as described under Materials and Methods.

$NaDodSO_4$ Gel Electrophoresis. The procedure described by Laemmli (1970) was used for $NaDodSO_4$ gel electrophoresis.

Radioactivity Determinations. Scintillation fluid was prepared by mixing 160 mL of Liquifluor (New England Nuclear, Inc.) with 3.8 L of toluene. Samples were counted in a Beckman LS300 liquid scintillation counter, which had a 33% counting efficiency, as determined with [^{3}H]toluene of known specific activity.

Results

Binding of [^{3}H]Dihydrotestosterone 17β-Bromoacetate to the Androgen Receptor. In order to determine whether the nonradioactive affinity label would compete with dihydrotestosterone for the active binding site on the androgen receptor, we incubated rat prostate cytosol with [^{3}H]dihydrotestosterone and increasing concentrations of either dihydrotestosterone or dihydrotestosterone 17β-bromoacetate. Figure 2 shows the competition by both steroids. Dihydrotestosterone competed with [^{3}H]dihydrotestosterone for the binding site in a dose-dependent manner. Fifty percent of the binding activity was competed with 0.5 nM dihydrotestosterone, which is consistent with the dissociation constant of the dihydrotestosterone–receptor complex (Liao et al., 1975). Dihydrotestosterone 17β-bromoacetate also competed with [^{3}H]dihydrotestosterone in a dose-dependent manner. Approximately 15 nM of dihydrotestosterone 17β-bromoacetate was required to compete for 50% of the binding sites, indicating that dihydrotestosterone 17β-bromoacetate has less affinity than dihydrotestosterone for the binding site. Nonetheless, 100% of the [^{3}H]dihydrotestosterone was displaced by the affinity label at higher concentrations (4 μM).

The time course of [^{3}H]dihydrotestosterone 17β-bromoacetate binding in prostate cytosol was measured over a period of 3 h at 0 °C. Half-maximum binding was achieved at approximately 4 min after initial incubation. Maximum binding was obtained at approximately 60 min, and thereafter a plateau was reached. The rate of association (k_a) during the initial 30 min was determined by the following equation (Nakahara & Birnbaumer, 1974):

$$1 - \frac{[RS](k_a[S] + k_i)}{R_t k_a[S]} = e^{-(k_a[S]+k_d)t}$$

where [RS] is the receptor–steroid complex concentration, [S]

Table I: Binding Specificity of [^{3}H]Dihydrotestosterone 17β-Bromoacetate in Rat Prostate Cytosol

steroid	% inhibition[a]
dihydrotestosterone 17β-bromoacetate	100
dihydrotestosterone	100
testosterone	69
progesterone	<1
17β-estradiol	<1
cortisol	<1

[a] Rat prostate cytosol was prepared as described under Materials and Methods. The cytosol was incubated with or without 125 nM unlabeled competing steroids. After 4 h of incubation at 0 °C, 12.5 nM [^{3}H]dihydrotestosterone 17β-bromoacetate was added to all tubes. The incubation was continued for 3 h. Binding radioactivity was determined by charcoal binding assay as described under Materials and Methods.

is the steroid concentration, k_i is the rate of inactivation, R_t is total receptor sites determined from Scatchard analysis, k_d is the dissociation rate, and t is time. Both dissociation and inactivation of the steroid–receptor complex were assumed to be negligible during this time period, and therefore, k_d and k_i were made zero. The rate of association was $0.05 \times 10^9 M^{-1}$ h^{-1} for [^{3}H]dihydrotestosterone 17β-bromoacetate and $0.3 \times 10^9 M^{-1}$ h^{-1} for [^{3}H]dihydrotestosterone (data not shown). The faster rate of association with [^{3}H]dihydrotestosterone than with [^{3}H]dihydrotestosterone 17β-bromoacetate is consistent with the higher affinity of [^{3}H]dihydrotestosterone for the receptor as shown in Figure 2.

The steroid specificity of the [^{3}H]dihydrotestosterone 17β-bromoacetate binding was investigated (Table I). Both dihydrotestosterone 17β-bromoacetate and dihydrotestosterone competed with 100% of the radioactive dihydrotestosterone 17β-bromoacetate for the receptor binding site at the concentrations tested. Testosterone competed less well (69%). Neither progesterone, 17β-estradiol, nor cortisol was able to compete. These data suggest that the affinity label binds to the active site of the androgen receptor in a structure-specific manner.

One of the functional properties of the androgen receptor is its ability to bind to DNA after transformation. The androgen receptor has been shown to undergo transformation to a DNA binding state after warming, ammonium sulfate precipitation, or salt treatment (Chang et al., 1982, 1983; Chang & Tindall, 1983; Liao et al., 1975). The following experiment was designed to determine if the affinity-labeled receptor could be transformed to a DNA binding state by heat treatment. Prostate cytosol was passed through a DNA–Sepharose column. The flow-through fractions were labeled with either [^{3}H]dihydrotestosterone 17β-bromoacetate or [^{3}H]dihydrotestosterone for 1 h at 0 °C. Each sample was incubated at 23 °C for 30 min and applied to a DNA–Sepharose column. The bound radioactivity was eluted from the column with TEDN buffer. The affinity-labeled complexes were eluted from the DNA column in a region corresponding to the transformed [^{3}H]dihydrotestosterone–receptor complexes (data not shown). These results suggest that the androgen receptor can be transformed by heat after binding to the affinity label.

Evidence of Covalent Attachment. Two criteria were chosen to test whether covalent attachment existed between [^{3}H]-dihydrotestosterone 17β-bromoacetate and the androgen receptor: (1) resistance to treatment with organic solvent and (2) irreversible binding under nondenaturing conditions.

Experiments were performed to determine if specific covalent binding remains after trichloroacetate precipitation and treatment with organic solvents. One set of tubes containing either [^{3}H]dihydrotestosterone 17β-bromoacetate or [^{3}H]dihydrotestosterone was incubated at 0 °C for 3 h while another set was incubated at 0 °C for 2.5 h and then 23 °C for 0.5 h. Half of each set was subjected to a charcoal binding assay, while the other half was precipitated with trichloroacetate and then extracted with ether. Table II shows the radioactivity remaining after either charcoal or organic solvent treatment. Results from the charcoal binding assay of the nondenatured receptor revealed a 40% reduction in specific affinity-labeled binding activity after a 0.5-h incubation at 23 °C. This reduction in specific binding was due to increased nonspecific binding during incubation. In contrast, when the same affinity-labeled receptor complexes were treated with trichloroacetate and ether, this additional 30-min incubation at 23 °C increased the specific covalent binding by 31%. Control experiments using [^{3}H]dihydrotestosterone exhibited a 16% increase in specific binding activity by the charcoal binding assay of the nondenatured receptor. However, only background radioactivity was observed after treatments with trichloroacetate and ether, indicating that no covalent bonds were formed between [^{3}H]dihydrotestosterone and the receptor. Covalent binding was linear up to 64 nM [^{3}H]dihydrotestosterone 17β-bromoacetate (data not shown), indicating that binding of the affinity label was concentration dependent as well. This agrees with the binding study described in Figure 2 which showed that approximately 4 μM of the affinity label is needed to saturate all the [^{3}H]dihydrotestosterone binding sites.

Table II: Covalent Binding of [^{3}H]Dihydrotestosterone 17β-Bromoacetate–Receptor Complexes in Rat Prostate Cytosol

	specific binding (cpm)	
	after charcoal	after organic solvent
dihydrotestosterone 17β-bromoacetate[a]		
(1) 3 h at 0 °C	50 483	9 149
(2) 2.5 h at 0 °C plus 0.5 h at 23 °C	29 086	11 972
dihydrotestosterone[b]		
(1) 3 h at 0 °C	36 000	257
(2) 2.5 h at 0 °C plus 0.5 h at 23 °C	41 630	356

[a] Rat prostate cytosol was saturated with unlabeled testosterone (16 nM) for 2 h at 0 °C before being applied to a DNA–Sepharose column. The flow-through fractions were pooled and precipitated with 40% ammonium sulfate. The pellet was resuspended in PE buffer. The sample was incubated with 16 nM [^{3}H]dihydrotestosterone 17β-bromoacetate ± 1.6 μM dihydrotestosterone 17β-bromoacetate either at 0 °C for 3 h or at 0 °C for 2.5 h plus 0.5 h at 23 °C. At the end of the incubation, samples were either treated with a solution containing 1% charcoal or precipitated with 10% trichloroacetate followed by extraction with ether. [b] The pellet from 40% ammonium sulfate was resuspended in PE buffer as described above except unlabeled testosterone was not added. The suspension was incubated with 16 nM [^{3}H]dihydrotestosterone ± 1.6 μM dihydrotestosterone either at 0 °C for 3 h or at 0 °C for 2.5 h plus 0.5 h at 23 °C. The samples were assayed as described in footnote *a*.

If the [^{3}H]dihydrotestosterone 17β-bromoacetate is binding covalently to the active site of the receptor molecule, then it should be possible to saturate the available binding sites with nonradioactive affinity label and prevent further binding of [^{3}H]dihydrotestosterone to the receptor under exchange conditions. We therefore incubated a receptor preparation with 4 μM dihydrotestosterone 17β-bromoacetate overnight at 0 °C and then attempted to exchange [^{3}H]dihydrotestosterone onto the receptor at 30 °C after removing excess unlabeled affinity label by charcoal treatment. It can be seen in Figure 3 that little or no [^{3}H]dihydrotestosterone could be exchanged onto the affinity-labeled receptor for up to 120 min at 30 °C.

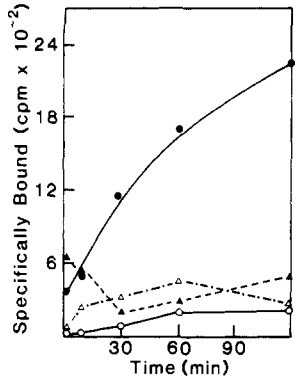

FIGURE 3: Exchange rate of steroids after incubating receptor with affinity label. Prostate cytosol was precipitated with 40% ammonium sulfate. After centrifugation, the pellet was resuspended in PE buffer. The solution was divided into two fractions: one fraction was treated with 16 nM dihydrotestosterone (circles), and the other one was treated with 4 μM dihydrotestosterone 17β-bromoacetate (triangles) at 0 °C for 16 h. At the end of the incubation, samples from both groups were treated with equal volumes of dextran-coated charcoal. After centrifugation, aliquots of the supernatant fluid were incubated with 16 nM [^{3}H]dihydrotestosterone in the presence or absence of 4 μM dihydrotestosterone either at 0 °C (open circles or triangles) or at 30 °C (closed circles or triangles) for various lengths of time: 1, 10, 30, 60, and 120 min. Specifically bound activity in each sample was determined by charcoal binding assay as described under Materials and Methods.

In contrast, [^{3}H]dihydrotestosterone could easily be exchanged at 30 °C onto receptor in samples which had been labeled with saturating amounts of dihydrotestosterone overnight. Control samples maintained at 0 °C exhibited no exchangeable binding activity, when preincubated with either the affinity label or dihydrotestosterone. These results add further evidence that dihydrotestosterone 17β-bromoacetate is binding covalently to the active site of the nondenatured receptor.

Molecular Properties of the [^{3}H]Dihydrotestosterone 17β-Bromoacetate–Androgen Receptor Complex. In order to determine if the protein which bound the affinity label was in fact the androgen receptor, we examined a number of physicochemical properties of the binding complex and compared them with the dihydrotestosterone–receptor complex. First, the Stokes radius of the receptor was examined. Cytosol from rat prostate was precipitated with 40% ammonium sulfate, incubated with [^{3}H]dihydrotestosterone 17β-bromoacetate, and applied to a gel filtration column. Each fraction was assayed for covalent binding by precipitation with trichloroacetate and extraction with methanol. It can be seen in Figure 4 that two major peaks of radioactivity were eluted from the column. One peak, which may represent an aggregated form of the receptor, coeluted with blue dextran (void volume), and a second peak eluted at a Stokes radius of 4.2 nm. The same Stokes radius was found when rat prostate cytosol was labeled with [^{3}H]dihydrotestosterone (Chang et al., 1983).

We next investigated the sedimentation properties of the affinity-labeled complex. An ammonium sulfate precipitated receptor preparation was labeled with [^{3}H]dihydrotestosterone 17β-bromoacetate and centrifuged through a 2–20% sucrose gradient in TEDGN buffer. The gradient tubes were fractionated, and each fraction was precipitated with trichloroacetate and extracted with methanol. Figure 6 shows that the receptor complexes sedimented as a major component at 4.5 S and a minor component at 9 S. The 4.5S peak of radioactivity could be competed out in the presence of excess unlabeled dihydrotestosterone. Under these conditions specific [^{3}H]dihydrotestosterone-labeled receptor complexes sedimented as one peak at 4.5 S (Figure 5). When salt was removed from the gradient, only one peak of activity sedimenting at 9 S was observed (data not shown). These hydrodynamic properties (Stokes radius and sedimentation coefficient) were used to calculate a molecular weight of 85 000 for the affinity-labeled receptor.

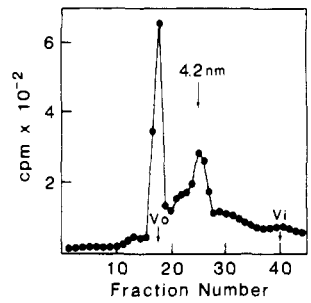

FIGURE 4: Gel filtration of [^{3}H]dihydrotestosterone 17β-bromoacetate bound complexes in rat prostate cytosol. An aliquot (3 mL) of prostate cytosol was precipitated with 40% ammonium sulfate and resuspended in 500 μL of PE buffer. The suspension was incubated with 16 nM [^{3}H]dihydrotestosterone 17β-bromoacetate for 16 h at 0 °C and applied to an agarose A-0.5m column (1.8 × 50 cm) which had been equilibrated with TEDN buffer. The column was calibrated with blue dextran (V_0) and 10 mg/mL each of the following proteins: bovine γ-globulin (5.2 nm) and bovine serum albumin (3.6 nm). V_i was determined by the radioactivity of the unbound [^{3}H]dihydrotestosterone 17β-bromoacetate peak. Two-milliliter fractions were collected under a 15-cm hydrostatic pressure. Each fraction was precipitated with 10% trichloroacetate and washed with 1 mL of methanol at room temperature before counting for radioactivity.

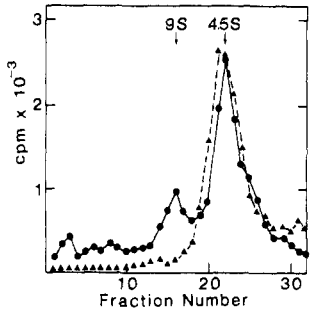

FIGURE 5: Sedimentation of covalently bound [^{3}H]dihydrotestosterone 17β-bromoacetate–receptor complexes. Rat prostate cytosol was precipitated with 40% ammonium sulfate. The pellet was resuspended in 500 μL of PE buffer. The solution was labeled either with 16 nM [^{3}H]dihydrotestosterone (closed triangles) or 64 nM [^{3}H]dihydrotestosterone 17β-bromoacetate (closed circles) at 0 °C for 2.5 h and 30 °C for 0.5 h. After cooling, samples were applied to 2–20% sucrose gradients in TEDGN buffer and centrifuged at 170000g for 18 h at 0–2 °C. Bovine liver catalase (11.3 S), bovine γ-globulin (6.9 S), bovine serum albumin (4.4 S), and myoglobin (2.0 S) were used as internal standards. The [^{3}H]dihydrotestosterone 17β-bromoacetate labeled fractions were assayed for covalent binding by sequential treatments with trichloroacetate and methanol. The [^{3}H]dihydrotestosterone-labeled fractions were assayed with charcoal binding assay as described under Materials and Methods.

In order to confirm the molecular weight of the receptor, we analyzed an affinity-labeled receptor preparation by gel electrophoresis under denaturing conditions. Due to the relatively low concentration of receptor binding sites in cytosol, a partially purified preparation was used for gel electrophoresis. The receptor preparation was equilibrated with [^{3}H]dihydrotestosterone 17β-bromoacetate for 2.5 h at 0 °C and then warmed at 23 °C for 30 min. The incubate was cooled in an ice bath (0 °C) and precipitated with 10% trichloroacetate. The precipitant was resuspended in sample buffer and electrophoresed through a $NaDodSO_4$ gel. After sequential washing of the gel with 40% methanol and 7% acetic acid, the gel was dried and subjected to fluorographic analysis and

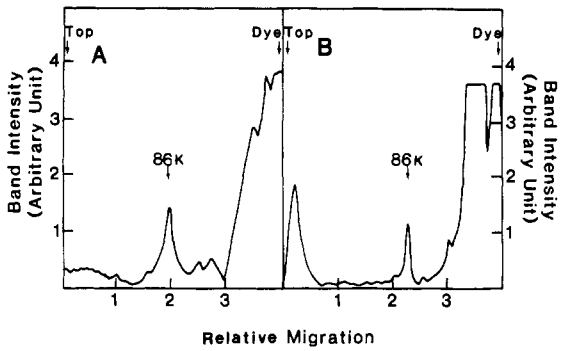

FIGURE 6: Gel scans of specifically bound [^{3}H]dihydrotestosterone 17β-bromoacetate–receptor complexes after NaDodSO$_4$ gel electrophoresis. Receptors were partially purified (approximately 5000-fold) by differential DNA–Sepharose chromatography and testosterone affinity chromatography as described previously (Chang et al., 1982, 1983). The receptor preparations were incubated with 10 nM [^{3}H]dihydrotestosterone 17β-bromoacetate in the absence or presence of 4 μM labeled dihydrotestosterone 17β-bromoacetate. Samples were further incubated at 23 °C for 30 min. Receptors were precipitated with 10% trichloroacetate, redissolved in sample buffer, applied to a NaDodSO$_4$ gel, and electrophoresed. Fluorographic treatment of the gels was performed as described previously (Chang et al., 1982, 1983). Gel scanning was performed on a densitometer using dual channels on both sample lanes. The resulting scanning curves represent specifically bound radioactivity of the [^{3}H]dihydrotestosterone 17β-bromoacetate–receptor complexes. The receptor preparations from rat prostate and seminal vesicle are shown in panels A and B, respectively.

densitometer scanning. Figure 6A shows a peak of specific radioactivity corresponding to a molecular weight of 86 000 which was similar to the molecular weight (85 000) determined under nondenaturing conditions (see Figures 4 and 5). Nonradioactive dihydrotestosterone 17β-bromoacetate competed for this binding component. This binding component could also be competed with unlabeled dihydrotestosterone. Several labeled bands of smaller molecular weight (45 000–30 000) were also observed. However, these bands were variable among preparations in both intensity and displacement by nonradioactive ligand. When [^{3}H]dihydrotestosterone 17β-bromoacetate alone was electrophoresed in a similar gel, radioactivity was only observed at the dye front. When receptor preparations were incubated with [^{3}H]dihydrotestosterone, no radioactive bands were detected in the gel, indicating that this steroid dissociated when the receptor was denatured.

Previous studies (Wilson & French, 1976, 1979) have shown that the androgen receptor in rat seminal vesicle has physicochemical properties which are similar to those of the rat prostate. We therefore compared the binding proteins of seminal vesicle with prostate. A specific peak of radioactive dihydrotestosterone 17β-bromoacetate was observed at a molecular weight of 86 000 (Figure 6B), which corresponded to the same molecular weight species found in rat prostate (Figure 6A). Excess unlabeled dihydrotestosterone 17β-bromoacetate or dihydrotestosterone displaced the radioactive ligand from this band. Specific bound activity was also found at the beginning and bottom of the running gel.

Discussion

These data demonstrate that both unlabeled and tritium-labeled dihydrotestosterone 17β-bromoacetate have been synthesized and characterized. This compound bound both specifically and covalently to the active binding site of the receptor. We have used this affinity label to characterize the molecular weight of the receptor under both denaturing and nondenaturing conditions.

The unlabeled dihydrotestosterone 17β-bromoacetate was pure as judged by nuclear magnetic resonance spectroscopy, mass spectroscopy, infrared spectroscopy, and elemental analysis. Chromatography of the tritium-labeled compound on a thin-layer plate showed only one peak of radioactivity, which comigrated with the nonradioactive material.

The affinity label appeared to bind to the active binding site of the androgen receptor as judged by a number of criteria. First, it competed with dihydrotestosterone for binding to the nondenatured receptor. Second, binding to this compound was structure specific. Finally, the Stokes radius (4.2 nm) and sedimentation coefficient (4.5 S) of the affinity labeled receptor were identical with those (4.2 nm and 4.5 S, respectively) of the receptor bound to dihydrotestosterone, thus indicating the same molecular weight (86 000). This molecular weight is consistent with that (M_r 86 000) reported for the cytosolic androgen receptor from rat ventral prostate under nondenaturing conditions (Chang et al., 1983; Bruchovsky et al., 1975) and the purified receptor under both denaturing and nondenaturing conditions (Chang et al., 1983). Thus, the affinity label appears to bind to the same molecular component as that which binds dihydrotestosterone. Moreover, the affinity-labeled receptor could be transformed to a DNA-binding state by heat treatment, indicating that the affinity label did not alter the DNA binding properties of the receptor. Similarly, the glucocorticoid receptor, bound to the electrophilic affinity label dexamethasone 21-mesylate, also retained its DNA binding ability (Simons et al., 1983).

Our data suggest that dihydrotestosterone 17β-bromoacetate binds covalently to the androgen receptor. First, binding was resistant to treatments with organic acid (trichloroacetic acid), detergent (sodium dodecyl sulfate), and organic solvents (methanol and ether). Second, binding was irreversible since dihydrotestosterone was not capable of exchanging with the affinity label after it was bound to the receptor. Covalent binding of testosterone 17β-bromoacetate to the androgen receptor (Mainwaring & Johnson, 1980) and 11α- and 16α-bromoacetate derivatives of progesterone to the progesterone receptor (Holmes & Smith, 1983) also has been described recently.

Three electrophilic derivatives of steroid compounds have been used to react irreversibly with receptors, namely, bromoacetate, mesylate, and aziridine. As shown in this study, dihydrotestosterone 17β-bromoacetate appears to bind with the active binding site of the androgen receptor in a structure-specific manner. Specific binding of 11α- and 16α-(bromoacetoxy)progesterone, dexamethasone 21-mesylate, and tamoxifen aziridine to progesterone (Holmes & Smith, 1983), glucocorticoid (Simons et al., 1983; Weisz et al., 1983), and estrogen receptors (Katzenellenbogen et al., 1983), respectively, has been reported. Both dihydrotestosterone 17β-bromoacetate and dexamethasone 21-mesylate have approximately $^1/_{20}$th the affinity for receptors as compared to their respective precursors. Because of this the receptor protein needs to be partially purified prior to covalent attachment. In contrast tamoxifen aziridine binds estrogen receptor with high affinity compared to diethylstilbestrol, and therefore, crude cytosol could be used in these studies (Katzenellenbogen et al., 1983).

Our laboratories have been interested in studying the steroid structural requirements for binding to the androgen receptor, androgen binding protein, and sex-steroid binding protein (Lobl et al., 1980; Tindall et al., 1978a,b). We have suggested previously that the receptor binding site consists of a surface with an L-shaped crease extending over the front top side (β-face; $C_{1,11,12,17}$ edge) of the molecule (Lobl et al., 1980).

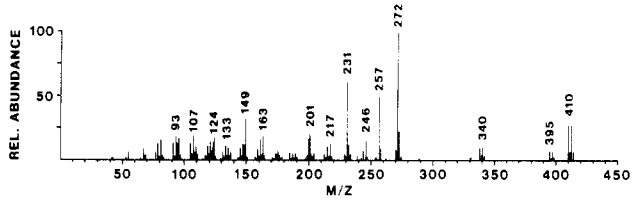

FIGURE 7: Mass spectrum of 17β-[(bromoacetyl)oxy]-5α-androstan-3-one. The mass spectrum confirms the molecular weight with intense ions at m/e 410 and 412. The base peak m/e 272 corresponds to the parent ion minus bromoacetic acid, and the intense peak at 257 suggests a further loss of a CH_3 radical. The remaining intense ions at the lower molecular weight are devoid from steroid skeletal fragments.

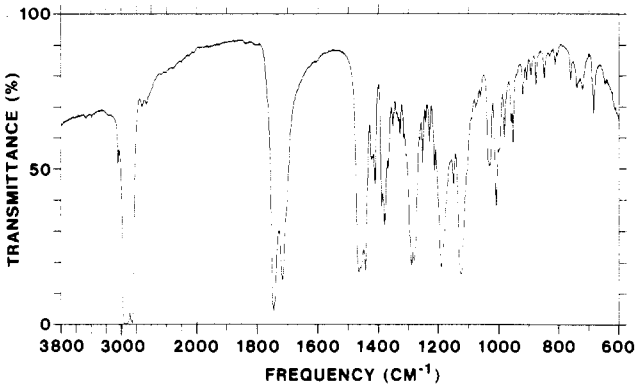

FIGURE 8: Infrared spectrum of 17β-[(bromoacetyl)oxy]-5α-androstan-3-one. The infrared spectrum is consistent with the desired structure. The C=O stretch at 1716 and 1745 cm^{-1} is assigned to the 3-ketone and the bromoacetate carbonyl, respectively. The fingerprint region is not remarkable in this case.

We are now in a position to use this affinity label to study the amino acid composition of the steroid binding site on the receptor. A number of progesterone and cortisone derivatives bearing alkylating functions have been used to determine the structure of the binding site of the enzyme, 20β-hydroxysteroid dehydrogenase (Gunguly & Warren, 1971; Sweet et al., 1972; Strickler et al., 1975). Most recently both 11α- and 16α-(bromoacetoxy)progesterone have been used to study the nucleophilic amino acid residues in or around the steroid binding site of the progesterone receptor (Holmes & Smith, 1983). It was found that 11α-(bromoacetoxy)progesterone alkylates the 1-position of a histidine residue, while 16α-(bromoacetoxy)progesterone alkylates the 3-position of histidine and a methionine residue. It is interesting to note that both bromoacetoxy derivatives (11α and 16α) of progesterone also alkylated the same amino acid residues of the enzyme, 20β-hydroxysteroid dehydrogenase (Sweet et al., 1972; Strickler et al., 1975). These observations strongly suggest a high selectivity of the bromoacetoxy derivatives of the steroid in binding with their respective nucleophilic amino acid residues in or around the macromolecular steroid binding site. Since the affinity label, dihydrotestosterone 17β-bromoacetate, has been shown to alkylate a carboxyl group of an aspartic or glutamic acid residue of the enzyme, 3α,20β-hydroxysteroid dehydrogenase (Sweet & Samant, 1980), it will be of interest to determine whether the same amino acid residues reside in or around the androgen receptor steroid binding site.

In conclusion, we have demonstrated that dihydrotestosterone 17β-bromoacetate binds covalently to the androgen receptor. Most importantly this compound can be used to characterize androgen receptors under both nondenaturing and denaturing conditions and represents a useful tool for future work with androgen receptor proteins and androphilic proteins in general.

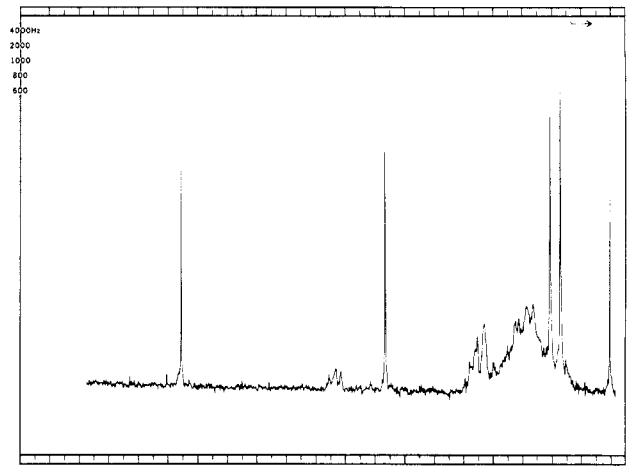

FIGURE 9: Nuclear magnetic resonance spectrum of 17β-[(bromoacetyl)oxy]-5α-androstan-3-one. The nuclear magnetic resonance spectrum is also consistent with the structure. The C-18 and C-19 angular methyl resonances were sharp singlets at δ 0.84 and 1.01, respectively. The bromoacetate methylene comes at δ 3.82 and the 17α-H is a triplet (J = 8 Hz) at δ 4.66. The remaining aliphatic region is not remarkable.

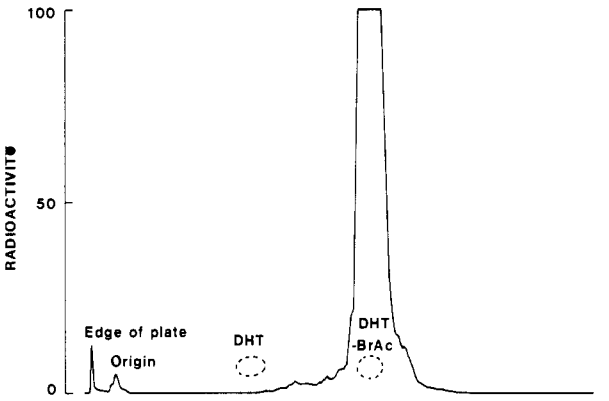

FIGURE 10: Thin-layer chromatogram of 17β-[(bromoacetyl)oxy]-[1,2,4,5,6,7,16,17-$^3H_8$]-5α-androstan-3-one. The radioactive compound was chromatographed over a silica gel thin-layer plate and scanned for radioactivity as described under Materials and Methods. DHT, dihydrotestosterone; DHT BrAc, 17β-[(bromoacetyl)oxy]-5α-androstan-3-one.

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Appendix

Mass spectral, infrared, and nuclear magnetic resonance analyses of 17β-[(bromoacetyl)oxy]-5α-androstan-3-one are given in Figures 7–9, and a thin-layer chromatogram of the deuterated compound is given in Figure 10.

Registry No. 17β-[(Bromoacetyl)oxy]-5α-androstan-3-one, 66656-21-1; bromoacetyl bromide, 598-21-0; dihydrotestosterone, 521-18-6; 17β-[(bromoacetyl)oxy][1,2,4,5,6,7,16,17-$^3H_8$]-5α-androstan-3-one, 89710-20-3; [1,2,4,5,6,7,16,17-$^3H_8$]dihydrotestosterone, 89710-21-4.

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Pyrimidine Catabolism: Individual Characterization of the Three Sequential Enzymes with a New Assay†

Thomas W. Traut* and Steven Loechel

ABSTRACT: We have developed a one-dimensional thin-layer chromatography procedure that resolves the initial substrate uracil and its catabolic derivatives dihydrouracil, *N*-carbamoyl-β-alanine (NCBA) and β-alanine. This separation scheme also simplifies the preparation of the radioisotopes of *N*-carbamoyl-β-alanine and dihydrouracil. Combined, these methods make it possible to assay easily and unambiguously, jointly or individually, all three enzyme activities of uracil catabolism: dihydropyrimidine dehydrogenase, dihydropyrimidinase, and *N*-carbamoyl-β-alanine amidohydrolase. Earlier reports had presented data suggesting that these three enzyme activities were combined in a complex because they appeared to be controlled at a single genetic locus [Dagg, C. P., Coleman, D. L., & Fraser, G. M. (1964) *Genetics 49*, 979–989] and because they appeared able to channel metabolites [Barrett, H. W., Munavalli, S. N., & Newmark, P. (1964) *Biochim. Biophys. Acta 91*, 199–204]. Although the three enzymes from rat liver have similar sizes, with apparent molecular weights of 218 000 for dihydropyrimidine dehydrogenase, 226 000 for dihydropyrimidinase, and 234 000 for NCβA amidohydrolase, they are easily separated from each other. Kinetic studies show no evidence of substrate channeling and therefore do not support a model for an enzyme complex. The earlier reports may be explained by our studies on the amidohydrolase, which suggest that under certain conditions this enzyme may become the rate-limiting step in uracil catabolism.

The catabolism of pyrimidines proceeds in three sequential steps, as illustrated in Figure 1. The physiological importance of this pathway is indicated by whole animal studies with mice (Sonoda & Tatibana, 1978), where over 80% of orally ingested [2-^{14}C]uracil is degraded and excreted in 8 h, about 50% as $^{14}CO_2$ and the rest as dihydrouracil and *N*-carbamoyl-β-alanine (NCβA)[1] in urine. With [2-^{14}C]uracil as the initial substrate, it is easy to determine the presence of all three enzyme activities by measuring the production of $^{14}CO_2$. This approach has established the existence of this pathway in rat liver (Canellakis, 1957; Fritzson, 1957), mouse liver (Dagg et al., 1964), regenerating rat liver (Ferdinandus et al., 1971), and rat hepatomas (Weber et al., 1971) as well as in microorganisms such as *Escherichia coli* (Simaga & Kos, 1981) and *Euglena gracilis* (Wasternack & Reinbothe, 1977). Since the first enzyme of the pathway is an NADPH-dependent

† From the Department of Biochemistry, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514. *Received October 28, 1983.* Supported in part by funds from the American Cancer Society (IN-15V and BC-451) and the University Research Council and by a Medical Faculty grant.

[1] Abbreviations: NCβA, *N*-carbamoyl-β-alanine; pDAB, *p*-(dimethylamino)benzaldehyde; PMSF, phenylmethanesulfonyl fluoride; TLC, thin-layer chromatography; DHU, dihydrouracil; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; UMP, uridine 5′-phosphate.

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