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Photoaffinity labeling of rat steroid 5α-reductase (isozyme-1) by a benzophenone derivative of a 4-methyl-4-azasteroid

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[1,2-^{3}H]N-4(Benzylbenzoyl)-3-oxo-4-aza-4-methyl-5α-androstane-17β-carboxamide ([^{3}H]-4MABP) has been synthesized as a photoaffinity probe of the steroid-binding domain of rat steroid 5α-reductase isozyme-1 (5αR-1). Reversible binding of the probe to 5αR-1 in microsomal preparations yielded a reversible dissociation constant (K_d) of ~3 nM, whereas inhibition experiments indicated that the probe had a 50% inhibition concentration of 4.4 nM and was a competitive inhibitor of the enzyme ($K_i \approx 3$ nM) with respect to testosterone. SDS-PAGE analysis of microsomal, detergent-solubilized, and (6.5%) polyethylene glycol-precipitated fractions of 5αR-1 photolyzed with [^{3}H]4MABP in the presence of NADPH showed that the radioactivity was incorporated into a single protein band with a mass of 26 kDa (apparent molecular weight of 5αR-1). UV photolysis was accompanied by an irreversible loss in enzyme activity, consistent with its covalent modification. Increasing the time of UV irradiation and concentration of [^{3}H]4MABP indicated that the half-life and apparent K_d for its photo insertion were ~3 min and 7.5 nM, respectively. Photolysis in the presence of a 20-fold excess of N,N-*diethyl-4-aza-4-methyl-3-oxo-5α-androstane-17β-carboxamide or the 3-carboxysteroid SKF-105111 resulted in partial protection of 5αR-1 from the probe, whereas minimal incorporation of radioactivity was observed in the absence of NADPH or in the presence of NADP$^+$. The results indicate that [^{3}H]4MABP is an effective probe of the steroid (D-ring) binding domain of 5αR-1. (Steroids* **61**:323–331, 1996)

Keywords: 5α-reductase; rat; photolabeling; benzophenone; 4-azasteroid

Introduction

The NADPH-mediated Δ^4 reduction of 3-keto-Δ^4 steroids is catalyzed by a family of isozymes called 5α-reductases (5αR).[1,2] Recent cloning and expression studies[3–6] have demonstrated that humans, monkeys, and rats possess two isoforms (designated isozyme-1 and -2) that can be distinguished on the basis of substrate affinity, pH optima, and inhibition by certain 4-azasteroids. 5αR reduction of testosterone to the more potent androgen 5α-dihydrotestosterone (5α-DHT) in the prostate is important in male sexual differentiation,[7,8] since a deficiency of this enzyme leads to male pseudohermaphroditism.[7–11] Elevated levels of 5αR and 5α-DHT in the prostate have been implicated in the pathogenesis of benign prostatic hyperplasia and prostate cancer,[12,13] whereas overactivity of 5αR in peripheral tissues such as the skin leads to male pattern baldness,[14,15] acne,[16] and hirsutism among women.[17]

The membrane-bound nature of the enzyme has made its purification to homogeneity difficult. One recent report has described the purification of the human (prostatic) isozyme-2 by detergent extraction, followed by multiple chromatography steps.[18,19] However, to date, pure 5αR-1 has not been isolated from natural or recombinant sources. Studies of hybrid enzymes obtained from chimeric constructs of rat and human type-1 cDNAs[20] and naturally occurring mutants of the type-2 enzyme[21] have suggested putative do-

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mains involved in the interaction with cofactor, steroid substrate, and inhibitors. We recently described the photolabeling and subsequently identified the $NADP^+$ (adenine) binding domain of rat 5αR-1.[22,23] The isolated peptide was shown to reside in the most highly conserved region of the sequence, suggesting that the cofactor-binding domain is similar in all of the 5α-reductases. The steroid-binding domains, on the other hand, have not been directly identified, although the above-mentioned studies have suggested that residues important for steroid binding reside in the N-terminal portion of the sequence.

Two classes of synthetic steroids, namely the 4-azasteroid[24,25] and the 3-carboxy steroid acrylates,[26,27] have been most extensively studied as inhibitors of 5α-reductase.[28,29] Both classes of inhibitors have been designed based on putative transition state intermediates, resulting in potent inhibition of the enzyme. Both inhibitor classes displayed an absolute requirement for reduced or oxidized cofactor, resulting in the formation of ternary dead-end complexes.[28,29] Inhibition of 5αR by most 4-azasteroids was shown to be competitive with respect to the substrate, whereas the 3-carboxy steroids were uncompetitive inhibitors of the enzyme. The different isozymic forms were inhibited to different extents by the 4-azasteroids, depending on the substitution at the 17β position of the steroid D-ring and to some extent structural variation of the A-ring.[30] These differential reactivities led to the development of MK-906 (Finasteride), a potent inhibitor of human prostatic isozyme-2, which is currently used in the clinical treatment of benign prostatic hyperplasia.[31] In the current study we have synthesized a photoreactive benzophenone derivative of a 4-methyl-4-azasteroid as a probe of the steroid (D-ring) domain and have described its binding and photolabeling properties with rat 5αR-1.

Experimental

All chemicals were American Chemical Society reagent grade. [1,2,6,7-^{3}H]Testosterone ([^{3}H]T; 100 Ci/mmol) for measurement of 5αR-1 activity was obtained from Amersham Life Sciences (Arlington Heights, IL, USA). NADPH and $NADP^+$ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The 3-carboxysteroid SKF 105111 [17β-(*N,N*-diisopropyl-carbamoyl)-androst-3,5-diene-3-carboxylic acid] was a generous gift from Dr. Mark Levy (Department of Medicinal Chemistry, Smith Kline and Beecham Pharmaceuticals, King of Prussia, PA, USA). *N,N,*-Diethyl-4-aza-4-methyl-3-oxo-5α-androstane-17β-carboxamide (4MA) and 4-aza-4-methyl-5α-androst-1-en-3-one-17β-carboxylic acid were a generous gift from Dr. Gary Rasmusson (Department of Medicinal Chemistry, Merck, Sharp and Dohme Research Laboratories, Rahway, NJ, USA).

Microsomes were prepared from the livers of female Sprague-Dawley rats as described earlier.[22] Briefly, livers were homogenized into 20 mM sodium phosphate (pH 6.5) containing 0.25 M sucrose. This was centrifuged at 10,000 × *g* to remove nuclear and mitochondrial fractions, and the resultant supernatant was centrifuged for 60 min at 100,000 × *g*. The pellet was resuspended in the above buffer and stored at −80°C.

Microsomes containing 5αR-1 activity were detergent-solubilized by homogenizing the microsomal pellet in 100 mM sodium citrate (pH 7.5) containing 100 mM KCl, 0.4% lubrol, 20% glycerol, and 200 μM NADPH in a glass-teflon homogenizer, and stirring at 4°C for 30 min. The resulting suspension was centrifuged at 100,000 × *g* for 60 min, and the supernatant was saved and stored at −80°C.

Polyethylene glycol (PEG) fractionation was carried out by slow addition of a 50% solution of PEG-6000 to detergent-solubilized preparations (4–5 mg/ml) to a final concentration of 6.5%. The mixture was stirred at 4°C for 20 min and centrifuged at 10,000 × *g* for 20 min. The pellet was resuspended in 20 mM sodium citrate (pH 7.5) containing 20 mM KCl, 0.2% lubrol, 20% glycerol, and 100 μM NADPH such that the final concentration of protein was ~2 mg/ml and stored at −80°C.

5αR-1 activity was measured at 5 μM [^{3}H]T and 200 μM NADPH at 37°C, using the isocratic high-performance liquid chromatography (HPLC) system previously described.[22] Typically, activities of 12–16 nmol/min/mg of protein were obtained with the 6.5% PEG fraction. Protein concentrations were determined by the Biuret method.[32]

Synthesis of N-4(benzylbenzoyl)-3-oxo-4aza-4-methyl-5α-androstan-17β-carboxamide

4-Aza-4-methyl-5α-androstan-3-one-17β-carboxylic acid (4MACOOH) was synthesized from pregnenolone as described by Rasmusson et al.[24] The purity of intermediates and final product was verified by TLC, melting point, GC/MS and by ^{1}H NMR.

4-Aminomethylbenzophenone (4-aminomethyl-BP) was synthesized from 4-methyl-benzophenone as reported by Rajagopalan et al.[33] Briefly, 4-bromomethyl-BP was obtained by refluxing 4-methyl-BP with 1.1 equivalents of *N*-bromosuccinimide in the presence of 2,2′-azobisisobutyronitrile at 90°C. The product was extracted and treated with 1.1 equivalents of *N*-potassium pthalimide, and the reaction was quenched by pouring into water, followed by extraction into chloroform; the product was dried over $MgSO_4$. This was treated with 1.5 molar equivalents of hydrazine hydrate and refluxed in methanol, concentrated, treated with concentrated HCl, and extracted with ethyl acetate. The organic layer was washed with 5N HCl, and the combined aqueous layers were adjusted to pH 9–10 with NaOH. The product was extracted with chloroform, washed with saturated NaCl, dried over anhydrous Na_2SO_4, and rotovaped to dryness. Purity was confirmed by IR, ^{1}H NMR, ^{13}C NMR, and mass spectrometry. For prolonged storage the amine was converted to the hydrochloride (dry HCl) and kept in the dark at −20°C.

4MACOOH was conjugated to 4-aminomethyl-BP using the water-soluble carbodiimide 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide (EDC) (Aldrich). One hundred milligrams (0.3 mmol) of 4MACOOH was suspended in 2 mL Dimethyl Formamide (DMF), and 4-aminomethyl-BP (297 mg, 1.2 mmol) was added, followed by 0.1 mmol of pyridine and 0.6 g EDC in 1 mL 50% aqueous DMF. The mixture was flushed with N_2, covered, and allowed to stir at room temperature overnight (~14 h). The clear solution was diluted with ~4 mL water and extracted with ethyl acetate (2 × 2 mL). The organic layer was separated and washed with 0.1N HCl, water, saturated $NaHCO_3$, and saturated NaCl and dried over anhydrous Na_2SO_4. This was filtered and evaporated to dryness, and the solid material was resuspended in ethyl acetate and purified by preparative thin-layer chromatography (TLC) (silica gel 60 F_{254}, 20 × 20 cm; Merck) and developed with 65% $CHCl_3$/35% EtOAc. The R_f of the UV active band corresponding to the steroid conjugate was determined by cochromatography of a smaller sample, which was visualized using an ultraviolet lamp (254 nm). The main ultraviolet active band was scraped off, extracted in a column with Ethyl Acetate/Methanol (EtOAc/CH_3OH) (9:1), and filtered. This was dried and resuspended in a minimum volume of methanol. Purity was confirmed by TLC, HPLC, ultraviolet absorbance (Figure 1) and by Fast Atom Bombardment/Mass Spectroscopy (FAB/MS) in Nitrobenzyl Alcohol (NBA) (MH^+ = 527.5).

Radioactive 4MACOOH was prepared by tritium reduction of 4-aza-4-methyl-5α-androst-1-en-3-one-17β-carboxylic acid using a procedure identical to that reported by Liang et al.[34] Briefly, 40

mg of the precursor steroid was dissolved in DMF, and 10% Pd/C (20 mg) was added. The mixture was stirred under 1 atm of tritium gas for 2 h, filtered, and evaporated to dryness (specific activity, 14.4 mCi/mg; ~5 Ci/mmol). The custom tritium reduction was performed by New England Nuclear. Final purification of the [1,2-^{3}H]4MACOOH was carried out by TLC using a solvent system consisting of $CHCl_3$/isopropanol/88% HCOOH (90:18:3). The final specific activity was 5 mCi/μmol.

[^{3}H]4MACOOH (2.5 mCi, 0.5 μmol) was dissolved in 0.3 mL DMF, and then 0.5 μmol of pyridine (in 20 μL DMF) was added, followed by 10 μL of 0.5 mg/mL EDC (~13 μmol) in 50% aqueous DMF. The mixture was shaken at room temperature for 1 h, after which 10 mg (~50 μmol) 4-aminomethyl-BP was added. The mixture was shaken under N_2 in the dark for ~72 h, during which three additions of EDC were made, totalling ~35 μmol. The conversion of the free carboxylic acid to the BP conjugate was monitored by HPLC (C_{18}-Resolve 3.9 × 150 mm, Waters) using an isocratic elution system (CH_3CN/H_2O 48:52) and a flow rate of 1 ml/min. The conjugate eluted at ~22 min, versus the free acid at ~2 min. The mixture was diluted to 4 mL with water and extracted twice with 1 mL CH_2Cl_2. The organic layer was washed four times with 1 mL 0.1N HCl, 1 mL distilled water, 1 mL saturated $NaHCO_3$, and 1 mL saturated NaCl, dried over anhydrous Na_2SO_4, and evaporated to dryness. Final purification was achieved by HPLC using the isocratic elution system described above. The ultraviolet spectrum of [^{3}H]4MABP was identical to the unlabeled steroid conjugate (not shown). Typically, specific activities of ~5–6 Ci/mmol were obtained (concentration of the probe was determined assuming a molar absorptivity of 14,500 at 258 nm).

Reversible and irreversible binding of [^{3}H]4MABP to 5αR-1 in microsomes

Reversible binding of the probe to 5αR-1 in microsomes was investigated by incubation of ~50 μg microsomal protein with increasing concentrations of [^{3}H]4MABP for 45 min at 4°C in the presence or absence of 100 μM NADPH in 0.5 mL of 20 mM sodium phosphate buffer (pH 6.5). After the incubation period, the samples were deposited on polyvinyl difluoride (PVDF) membranes (25 mm, 0.22 μm; Millipore), followed by five washes (2 mL each) with sodium phosphate buffer containing 0.1% Tween-80 and 1 μM NADPH (NADPH was omitted in the wash for samples incubated in the absence of NADPH). Radioactivity on the filters was determined by scintillation counting in 10 mL Ultima Gold XR (Packard) liquid scintillation cocktail in a Packard scintillation counter. Reversibly bound radioactivity was estimated from the difference in the values obtained in the presence and absence of NADPH, and data were analyzed by the method of Scatchard.

Irreversible binding of [^{3}H]4MABP was carried out using a procedure similar to that described above, except that after incubation with [^{3}H]4MABP, samples were exposed to UV light for 15 min (254 nm, 4 mW/cm^2) at 4°C. After photolysis, 4MA was added to a final concentration of 20 μM, along with 100 μM $NADP^+$, followed by incubation for 45 min. The samples were deposited on filters and washed five times with 2 mL 20 mM phosphate buffer (pH 6.5) containing 0.1% Tween-80, 20 μM 4MA, and 100 μM $NADP^+$. Radioactivity on the filters was determined as described above.

Determination of IC_{50} and inhibition constant

Reversible inhibition of 5αR-1 was determined in 6.5% PEG-precipitated fractions at saturating levels of T (5 μM) and NADPH (200 μM) by measuring enzyme activities at 37°C after preincubation of the enzyme with increasing concentrations of [^{3}H]4MABP. Before measurement of enzyme activity, preparations of 5αR-1 were preincubated with the appropriate concentration of probe, T, and NADPH for 45 min (4°C) to ensure maximum binding of the steroid conjugate (which is at maximum after 30 min), followed by measurement of product at increasing time intervals between 0 and 8 min (37°C). The activity was estimated from the slope of a plot of product versus time. In the case of K_i determination, the enzyme activity was determined at different concentrations of [^{3}H]4MABP (0–20 nM) while varying the concentration of T (0.5–5 μM) at a fixed concentration of NADPH (200 μM). Data were analyzed by Lineweaver-Burke plots.

Photolabeling of 5αR-1

All photolabeling experiments were carried out at 4°C. Microsomal preparations (0.2 mL; ~1 mg/mL) were photolyzed in 20 mM sodium phosphate (pH 6.5) containing 0.01% Tween-80 and 100 μM NADPH. Detergent-solubilized and PEG-fractionated samples (1–2 mg/mL) were photolyzed in 20 mM sodium citrate (pH 7.5) containing 20 mM KCl, 0.2% lubrol, 20% glycerol (buffer A), and 100 μM NADPH. Samples were incubated with 1 μM [^{3}H]4MABP for 45 min before photolysis. Photolysis was carried out with a hand-held ultraviolet lamp (254 nm, 4 mW/cm^2) for 0–15 min. PEG-fractionated samples that were used in the time-dependent inactivation studies were incubated with 100 μM $NADP^+$ for 45 min after photolysis. The samples were precipitated with PEG-6000 and the supernatant was discarded. Incubation with $NADP^+$ and PEG precipitation were repeated four times after each resuspension to ensure the removal of reversibly bound [^{3}H]4MABP. Final resuspension was in buffer A, containing 100 μM NADPH. The samples were assayed for 5αR-1 activity and analyzed by SDS-PAGE, autofluorography, and gel-slicing techniques. Samples used in photolysis experiments conducted in the absence of NADPH or in the presence of $NADP^+$ were first PEG-precipitated and washed twice to remove excess cofactor. This was followed by resuspension in buffer A alone or buffer A + 100 μM $NADP^+$, and photolysis with the probe under conditions similar to those described above.

SDS-PAGE analysis of labeled fractions

Microsomal, detergent-solubilized, and PEG-fractionated samples were solubilized in protein-solubilizing mix (PSM) (125 mM Tris-HCl, pH 6.8, 4% SDS, 1.4 M β-mercaptoethanol, 20% glycerol, and bromophenol blue dye), and ~100 μg of each sample was loaded on a 10% polyacrylamide gel and electrophoresed together with prestained standards (BioRad). After electrophoresis, the gel was soaked in 1.0 M sodium salicylate (pH 5.7–6.0) for 30 min[35] and subsequently dried and exposed to preflashed X-ray film (Kodak X-OMAT AR) for 24–96 h.[36] Gel slicing was performed according to established procedures.[37] Lanes of interest were cut out and sliced into 2.5-mm slices, and each slice was placed in a screw-capped glass scintillation vial, followed by the addition of 0.25 mL 70% perchloric acid and 0.5 mL 30% H_2O_2. The samples were incubated in a water bath or oven at 60°C for at least 12 h, after which they were allowed to cool and counted in 10 mL Ultima Gold XR (Packard) in a Packard scintillation counter.

Results

The synthesis of 4MABP and [^{3}H]4MABP from the free carboxylic acid is a relatively straightforward conjugation reaction carried out in the presence of the water-soluble carbodiimide EDC (Figure 1A). The UV spectrum (Figure 1B) and mass spectral data are consistent with the conjugation of the benzophenone chromophore with the free carboxylic acid. The molar absorptivity (14,500 at 258 nm) of the probe was estimated by measuring the absorbance of a solution of 4MABP of known concentration (based on

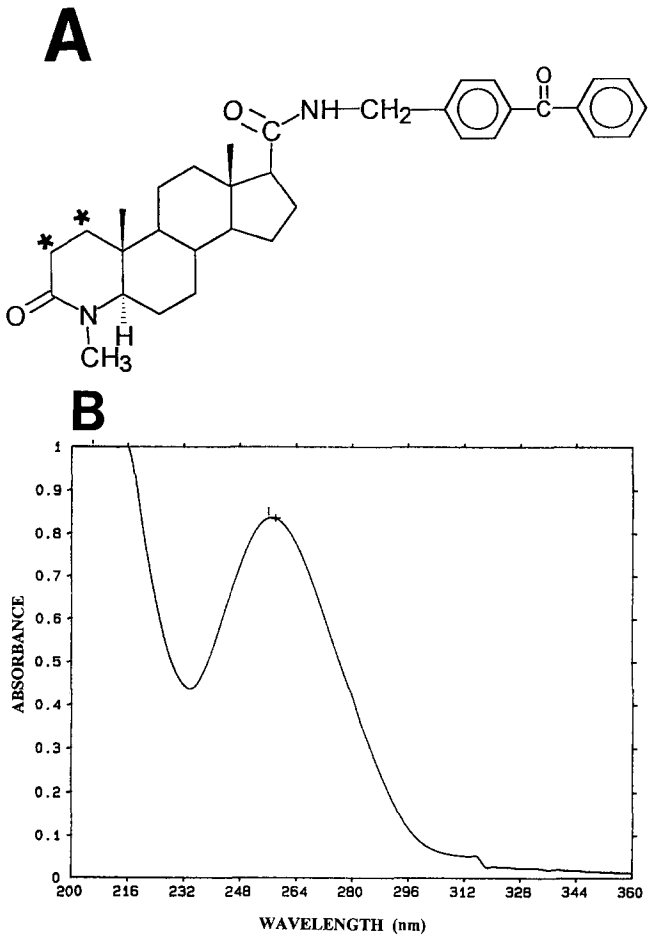

Figure 1 **A** Structure of [1,2-^{3}H]4MABP. Synthesis was achieved by conjugation of [1,2-^{3}H]4MACOOH to 4-aminomethyl-benzophenone in the presence of the water-soluble carbodiimide EDC, as described in the Experimental section. *, position of the tritium label. **B:** UV absorption spectrum of 4MABP. A molar absorptivity of 14,500 at 258 nm was estimated. All labeling experiments were performed by irradiating at 254 nm.

weight). Inhibition of 5αR-1 by the probe at saturating T and NADPH yielded a 50% inhibiting concentration of 4.4 nM, whereas Lineweaver-Burke analysis clearly indicated that the probe is a potent competitive inhibitor with respect to testosterone with a K_i of ~3 nM (data not shown). No inhibition of 5αR-1 was observed with 4-aminomethyl-BP alone at concentrations up to 1 μM (data not shown). The reversible binding of [^{3}H]4MABP to 5αR-1 was examined in microsomes (Figure 2A), and the results are consistent with saturable binding that is greater in the presence of NADPH, yielding a dissociation constant (K_d) of 3 nM. These results are similar to those obtained with other potent inhibitors of 5αR-1, such as 4MA, in that the binding is competitive and yields relatively low K_i values (K_i for 4 MA ≈ 9 nM).[34]

Irreversible binding of [^{3}H]4MABP was examined in microsomes; the results are shown in Figure 2B. Incubation of photolyzed microsomes with excess $NADP^+$ and 4MA, followed by filtering on PVDF membranes and extensive washing in the presence of $NADP^+$ and 4MA, ensured the removal of reversibly and nonspecifically bound probe. As in the case of reversible binding, the data were corrected for nonspecifically bound radioactivity by subtracting values obtained by photolysis in the absence of NADPH. An apparent K_d value of ~7.5 nM was obtained for photoincorporation of the probe into 5αR-1. The saturation data are consistent with site-specific labeling by [^{3}H]4MABP.

To determine the identity of proteins that are being labeled, microsomal, detergent-solubilized, and PEG-frac-

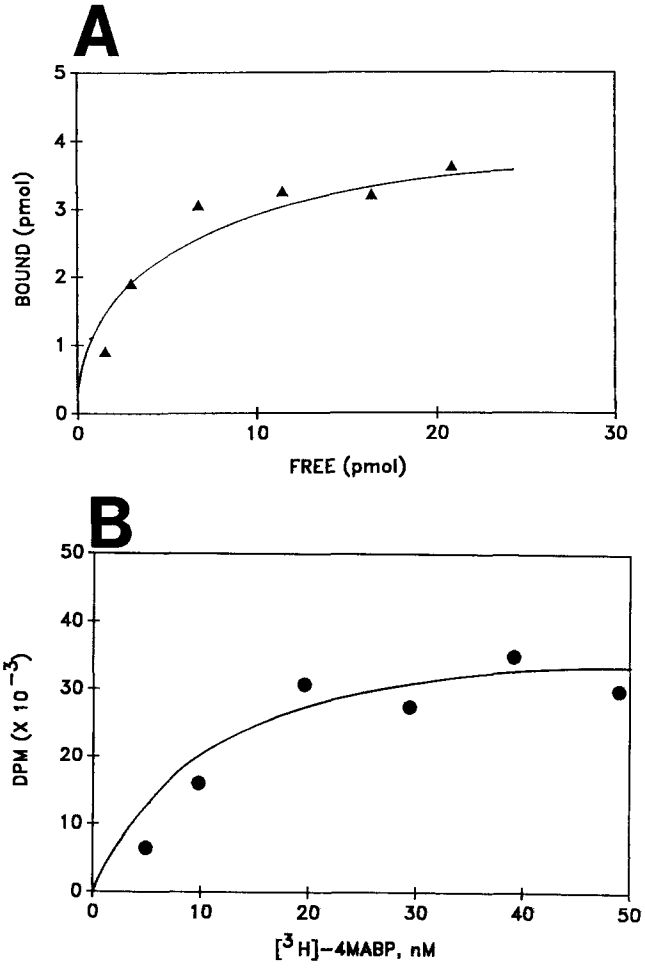

Figure 2 **A** Reversible binding of [^{3}H]4MABP to 5αR-1 in rat liver microsomes. Microsomes (0.05–0.1 mg/mL) were incubated with increasing concentrations of [^{3}H]4MABP (5–50 nM) in the presence of 100 μM NADPH, followed by precipitation on 0.22 μm PVDF membranes and several washed with buffer + 1 μM NADPH as described in the Experimental section. Filters were counted for radioactivity and the values were subtracted from those obtained by incubation in the absence of NADPH. Analysis was by the method of Scatchard. A K_d of ~3 nM was obtained. **B** Irreversible binding of [^{3}H]4MABP to microsomal 5αR-1. Microsomal samples (0.1 mg/mL) were preincubated with increasing concentrations (5–50 nM) of [^{3}H]4MABP in the presence of 100 μM NADPH for 45 min before photolysis of samples for 15 min (UV_{254}; 4 mW/cm^2). After photolysis, reversibly and nonspecifically bound probe was removed by incubation (45 min) with 20 μM 4MA and 1 mM $NADP^+$. Samples were deposited on PVDF membranes, washed with buffer containing 20 μM 4MA and 100 μM $NADP^+$, and counted for radioactivity. These values were subtracted from values obtained from a similar experiment conducted in the absence of NADPH. An apparent K_d of ~7.5 nM was obtained for covalent insertion of [^{3}H]4MABP.

tionated samples were incubated with 1 μM [^{3}H]4MABP in the presence of NADPH, followed by photolysis (254 nm) for 15 min. The samples were then incubated with excess 4MA and $NADP^+$ after photolysis, solubilized in PSM, and analyzed by 10% SDS-PAGE, followed by autofluorography. Figure 3 shows a typical autofluorogram obtained from such an experiment. Immediately apparent is the high degree of specificity of labeling by [^{3}H]4MABP. Only a single 26-kDa protein band was labeled by the probe in all of the preparations tested. The gel slicing data were also consistent with this interpretation (data not shown). No photoincorporation was observed in the absence of UV light (not shown).

The competitive inhibition of 5αR-1 by 4-azasteroids is dependent on the presence of NADPH, resulting in the formation of an enzyme/inhibitor/reduced cofactor ternary complex. The dependence of labeling on the presence of NADPH was determined by labeling of the enzyme in the presence and absence of NADPH and in the presence of $NADP^+$. Figure 4 shows a typical autofluorogram from such an experiment. It is clear that photoincorporation of the probe occurred only in the presence of NADPH. Only minimal amounts of radioactivity (<5%) were incorporated in the absence of NADPH or the presence of $NADP^+$ (lanes 4 and 5, Figure 4; see also Table 1). The specificity of incorporation was also tested in the presence of the competitive inhibitor 4MA, and the 3-carboxy steroid SKF 105111, which is a potent uncompetitive inhibitor of rat 5αR-1 ($K_i \approx$ 5–10 nM). Photolysis of PEG-fractionated samples of 5αR-1 with 1 μM [^{3}H]4MABP in the presence of 20 μM 4MA and 20 μM SKF 105111, followed by SDS-PAGE and autofluorography, showed that both steroids afforded partial protection against the probe (lanes 1, 2, and 3, Figure 4). This is an unexpected result, given the different natures of

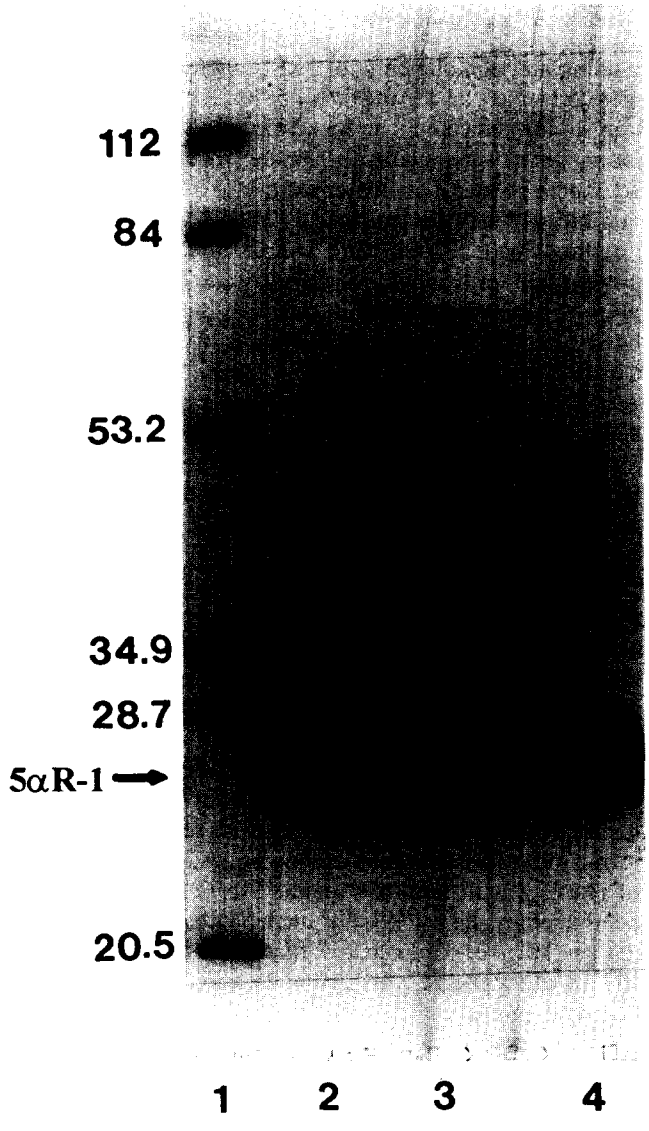

Figure 3 Autofluorography of [^{3}H]4MABP-labeled fractions. Microsomal **(lane 2)**, detergent-solubilized **(lane 3)**, and PEG-fractionated **(lane 4)** samples (1–2 mg/ml) were photolyzed with 1 μM [^{3}H]4MABP in the presence of 100 μM NADPH for 15 min followed by analysis on 10% SDS-PAGE and autofluorography as described in the Experimental section (exposure was for 72 h). Typically, 100 μg was loaded per lane. Markers on the left indicate the position of prestained molecular weight standards (BioRad).

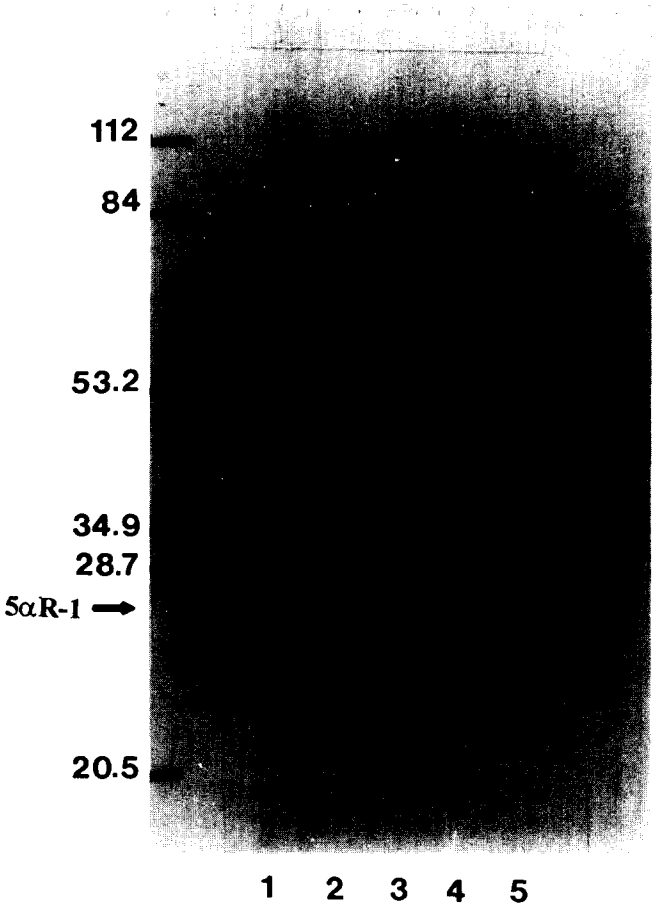

Figure 4 Effect of synthetic steroids on the photolabeling of 5αR-1 by [^{3}H]4MABP. PEG-fractionated samples (0.5 ml, 2 mg/ml) were photolyzed with 1 μM probe and subjected to 10% SDS-PAGE and autofluorograph (24 h), as described in the Experimental section. Lane assignments are as follows: **lane 1**: P5αR-1 + 1 μM [^{3}H]4MABP + 100 μM NADPH; **lane 2**: p5αR-1 + 1 μM [^{3}H]4MABP + 20 μM 4MA + 100 μM NADPH; **lane 3**: p5αR-1 + 1 μM [^{3}H]4MABP + 20 μM SKF 105111 + 100 μM NADPH; **lane 4**: P5αR-1 + 1 μM [^{3}H]4MABP; **lane 5**: P5αR-1 + 1 μM [^{3}H]4MABP + 100 μM $NADP^+$ (see Table 1). Approximately 100 μg protein was loaded per lane.

Table 1 Effect of synthetic steroids and cofactor on labeling of 5αR-1 by [^{3}H]4MABP

Sample	dpm/100 μg protein	Cross-linking efficiency (%)
P5αR-1 + 100 μM NADPH + 1 μM [^{3}H]4MABP + *hν*	2.3×10^5	30
P5αR-1 + 100 μM NADPH + 1 μM [^{3}H]4MABP + 20 μM 4MA + *hν*	9.9×10^4	12.8
P5αR-1 + 100 μM NADPH + 1 μM [^{3}H]4MABP + 20 μM SKF 105111 + *hν*	7.8×10^4	10.2
P5αR-1 + 1 μM [^{3}H]4MABP + *hν*	8.7×10^3	1.1
P5αR-1 + 1 μM [^{3}H]4MABP + 100 μM $NADP^+$ + *hν*	7.3×10^3	0.95

PEG-fractionated samples were incubated with the indicated compounds and then were photolyzed (*hν*: 254 nm for 15 min) followed by SDS-PAGE. Radioactivity in gel slices was determined as described under Experimental. Values represent radioactivity present in the 26-kDa band when 100 μg protein was loaded on the gel. The cross-linking efficiency of the probe was calculated assuming that 5αR-1 constitutes ~2% of the total protein in the PEG-fractionated sample. This value is estimated from inactivation and labeling studies with [2'-^{32}P]-2N_3-$NADP^+$.[22] The specific activity of the probe was ~5 Ci/mmol.

the inhibitions by the two classes of steroids. Table 1 shows the total bound radioactivity and the estimated cross-linking efficiency of the probe obtained from gel-slicing experiments.

The time-dependent inactivation of 5αR-1 activity was followed by irradiation of PEG-fractionated samples in the presence of 1 μM [^{3}H]4MABP at increasing times between 0 and 15 min. In these experiments, PEG-fractionated samples of the enzyme were incubated with 1 mM $NADP^+$ for 45 min after photolysis and precipitated with PEG to remove reversibly bound [^{3}H]4MABP. This wash procedure was repeated four times, after which samples were assayed for 5αR-1 activity. Control samples were treated in an identical manner to evaluate the effect of UV irradiation on the enzyme alone. Figure 5A shows results from a typical inactivation experiment. Approximately 40% inactivation (versus control) was observed after 15 min of UV irradiation, which is in reasonable agreement with the cross-linking efficiency of the probe (Table 1). The difference in activity at zero irradiation time is due to the presence of small amounts of reversibly bound 4MABP, which leads to inhibition of 5αR-1. A half-life for inactivation ($t_{1/2}$) of ~3 min was estimated from these data. A portion of each sample was solubilized in PSM and subjected to 10% SDS-PAGE, followed by analysis as before. The gel was cut out in the vicinity of the 5αR-1 band and dissolved, and radioactivity was estimated as described in the Experimental section. The values were corrected by subtracting background counts and plotted versus irradiation times (Figure 5B). The $t_{1/2}$ for photoincorporation of radioactivity (~3 min) is in close agreement with the value obtained with the inactivation data.

Discussion

We have previously demonstrated the photolabeling of the NADP(H) binding site of rat 5αR-1 by [2'-^{32}P]2-azido-$NADP^+$ and subsequently identified the NADP(H) (adenine) binding domain of the enzyme.[22,23] To date, the only photolabeling studies on the steroid-binding domain of the rat liver (5αR-1) and rat prostatic (5αR-2) enzymes were conducted, with [1,2-^{3}H]21-Diazo-4-aza-4-methyl-5α-androstan-3,20-dione ([^{3}H]diazo-MAPD) as the photoprobe.[34,38] In these studies NADPH-dependent reversible binding and photolabeling of 5αR-1 were observed, which were protected by the inclusion of excess 4MA. Gel filtration studies indicated that the labeled protein had a molecular weight of ~50 kDa under nondenaturing conditions. No attempt was made to analyze the labeled fractions under denaturing conditions, such as by SDS-PAGE. Thus, it was unclear whether the labeled species was a dimer, single polypeptide, or a subunit of the enzyme. In the case of the rat prostatic enzyme (5αR-2), labeling of a 20-kDa protein band was observed, as demonstrated by SDS-PAGE and autofluorography. Data with recombinant 5αR-2 have suggested that its apparent molecular mass on SDS-PAGE is approximately 23 kDa, although sequence data suggest a mass of 28–29 kDa. The discrepancy between the molecular weights of the labeled versus recombinant protein is not clear, although this may be due in part to the electrophoresis conditions used.

Benzophenone photophores have been widely used in the site-specific covalent modification of proteins.[33,39] They are chemically stable and can be excited at near-UV wavelengths (>300 nm) such that potential photochemical damage to proteins may be avoided. UV excitation of the carbonyl group results in a $n \rightarrow \pi^*$ transition and subsequent formation of a triplet keto-diradical, which is capable of hydrogen abstraction or electron transfer reactions involving nearby residues, leading to radical recombination and formation of stable carbon-carbon bonds. They are capable of reacting with relatively unreactive C-H bonds and are unaffected by the presence of nucleophiles and solvent water. Unlike other photoreactive functions, such as diazo-keto and aromatic azides, which photodecompose on irradiation, benzophenone chromopores can be re-excited several times after returning to the ground state, until photoinsertion in a nearby residue occurs.

The reversible binding data shown in Figure 2A, together with the inhibition data, suggest that 4MABP is a potent inhibitor of 5αR-1 and is similar to other 17β-substituted 4-azasteroids in that it is a competitive inhibitor of the enzyme with respect to substrate and that binding only occurs in the presence of reduced cofactor. Although the 17β-benzylbenzoyl substitution is bulky compared to some of the known 4-azasteroidal inhibitors, these results demonstrate that the enzyme displays tolerance for bulky groups at

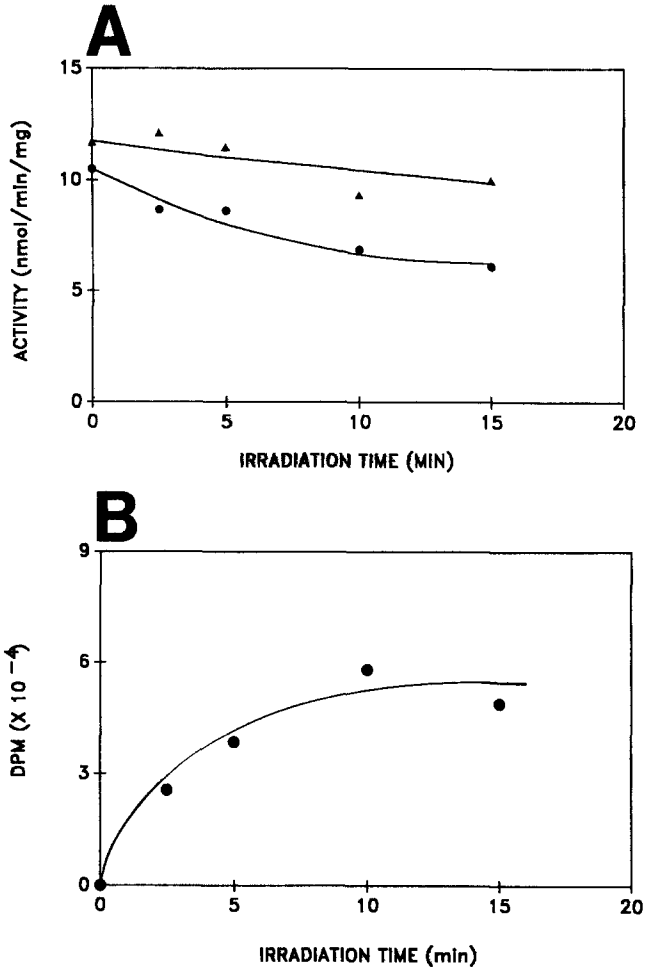

Figure 5 **A** Time-dependent inactivation of 5αR-1 by [^{3}H]4MABP. PEG-fractionated samples (2 mg/mL, 0.5 mL) were photolyzed (254 nm) in the presence of 1 μM [^{3}H]4MABP and 100 μM NADPH for the times indicated. The samples were precipitated with PEG-6000 (at a final concentration of 6.5%) and resuspended four times to remove reversibly bound [^{3}H]4MABP, and typically 5 μg protein was used to assay for 5αR-1 activity. Control samples were irradiated in the absence of [^{3}H]4MABP and treated in an identical manner. ●, P5αR-1 + 1 μM [^{3}H]4MABP + *hν*; ▲, P5αR-1 + *hν*. Approximately 40% inhibition was obtained under these conditions. The $t_{1/2}$ for inactivation was ~3 min. A cross-linking efficiency of ~30% was estimated for the probe under these conditions. **B** Time-dependent incorporation of [^{3}H]4MABP into 5αR-1. A portion of the photolyzed samples described in **A** were subjected to 10% SDS-PAGE. The gel was cut out in the vicinity of the 5αR-1 band and dissolved using procedures described in the Experimental section, followed by liquid scintillation counting. The peak counts were subtracted from the gel background counts and plotted versus irradiation times. The $t_{1/2}$ for incorporation of radioactivity was estimated to be ~3 min.

this position, suggesting that 5αR-1 may contain a hydrophobic cleft to accommodate such structures. Photolysis of [^{3}H]4MABP with microsomal preparations of 5αR-1, followed by treatment with excess 4MA and NADP$^+$, resulted in a substantial amount of the radioactivity remaining bound to the microsomes. Once again, the binding is dependent on the presence of NADPH and is saturable, indicative of site-specific labeling. An apparent K_d of ~7.5 nM was obtained for this process (Figure 2B).

Photolysis of microsomal, detergent-solubilized, and PEG-fractionated samples with [^{3}H]4MABP in the presence of NADPH, followed by analysis of the photolyzed fractions by SDS-PAGE, autofluorography, and gel slicing, showed the labeling of a single protein of mass 26 kDa, consistent with the apparent molecular mass of 5αR-1 under the conditions used (Figure 3). No labeling was apparent in the absence of UV light. Hiipakka et al.[40] have demonstrated from immunochemical studies that native 5αR-1 migrates with a molecular mass of 26 kDa. Our own studies on the photolabeling of 5αR-1 by [2′-^{32}P]2N$_3$-NADP$^+$ have confirmed this finding, and we subsequently identified the NADP(H)-binding domain of the labeled (26 kDa) 5αR-1 molecule. However, these results contrast somewhat with labeling studies with the NADP$^+$ analog in that, in the latter case, more than one protein (including 5αR-1) was ^{32}P labeled, demonstrating the lack of specificity of this probe.[22,23] In the current investigation [^{3}H]4MABP labeled only a single protein in all preparations tested, demonstrating its high degree of specificity.

The specificity of the labeling was further tested by carrying out photolysis in the absence of NADPH, in the presence of excess NADP$^+$, and by competition experiments in which photolabeling was carried out in the presence of the competitive inhibitor 4MA and the 3-carboxy steroid SKF 105111 (uncompetitive inhibitor). When photolabeling was carried out in the absence of NADPH or in the presence of NADP$^+$, only minimal amounts of radioactivity were incorporated, as demonstrated by the gel slicing data in Table 1 and the autofluorograph shown in Figure 4. The autofluorograph shown in Figure 4 also demonstrates the photolabeling of 5αR-1 by [^{3}H]4MABP in the presence of a 20-fold excess of 4MA. The data clearly showed that the 26-kDa band is partially protected from labeling by the probe, and this result is supported by the total radioactivity incorporated into 5αR-1 from the gel slicing data shown in Table 1. Protection was also observed in the presence of an excess (20-fold) of the 3-carboxy steroid SKF 105111, a potent uncompetitive inhibitor of the enzyme (Figure 4). This is a surprising result given the difference in the nature of the inhibition between these two classes of steroids (i.e., competitive versus uncompetitive). This suggests that the structure of the ternary complex between inhibitor/enzyme and cofactor should be different. One possible explanation for the observed data is that there is a significant overlap in the steroid binding domains (at least in the D-ring portion) of the two classes of steroids, resulting in partial protection from the probe.

Photodependent inactivation of 5αR-1 was also followed as a function of increasing irradiation time; the results are summarized in Figure 5A. Increasing the irradiation time led to a time-dependent decrease in the 5αR-1 activity, which appeared to reach maximum by 15 min (~40% versus control). A cross-linking efficiency of ~30% was estimated from the gel slicing data shown in Table 1. UV irradiation alone results in a smaller decrease in the enzyme activity probably due to slower photodegradation of the enzyme. A half-life for photoinactivation of ~3 min was estimated from these data. The half-life for photoincorporation of the probe was also determined by analysis of irradiated samples by

SDS-PAGE, autofluorography, and gel slicing. The gel-slicing data shown in Figure 5B clearly demonstrate a time-dependent increase in labeling of 5αR-1. A half-life of ~3 min was estimated for this process, a value that is in agreement with the $t_{1/2}$ for photoinactivation. Although the photolabeling data shown here were obtained using relatively short wavelengths (254 nm), photoincorporation was also achieved either with a 300-nm cutoff filter on the existing UV source or with a high-intensity (Hanovia 450-w) mercury vapor lamp with a 300-nm pyrex filter. However, the two sources required prolonged irradiation times to achieve similar levels of incorporation and did not significantly decrease the extent of photoinactivation of the enzyme by light alone.

In summary, all preparations containing 5αR-1 activity were labeled by [^{3}H]4MABP, in that they specifically incorporated ^{3}H upon UV exposure, resulting in a decrease in enzyme activity consistent with its covalent insertion. The low K_d values and $t_{1/2}$ for photoinsertion, together with its high specificity, make it an effective probe of the steroid (D-ring) binding domain. Future studies will focus on the purification of [^{3}H]4MABP-labeled 5αR-1, followed by isolation of the labeled peptide(s) and sequence analysis. Such studies are currently under way in this laboratory.

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