Identification of the NADP(H) Binding Site of Rat Liver Microsomal 5α-Reductase (Isozyme-1): Purification of a Photolabeled Peptide Corresponding to the Adenine Binding Domain†

Anjan K. Bhattacharyya,*,‡ Ashok J. Chavan,§ Boyd E. Haley,§ Matthew F. Taylor,|| and Delwood C. Collins‡

Departments of OB/GYN, Medicinal Chemistry, and Biochemistry, Colleges of Medicine and Pharmacy, University of Kentucky Lucille P. Markey Cancer Center, and VA Medical Center, Lexington, Kentucky 40506

Received September 9, 1994; Revised Manuscript Received November 11, 1994⊗

ABSTRACT: We have previously shown that [2′-^{32}P]-2-azido-NADP$^+$ is an effective probe of the NADP-(H) binding site of rat liver microsomal 5α-reductase (5αR-1) [Bhattacharyya et al. (1994) *Steroids 59,* 634−641]. PEG-fractionated (6.5%) detergent-solubilized preparations (40 mg) containing 5αR-1 activity were UV-photolyzed with [^{32}P]-2-azido-NADP$^+$ and subjected to preparative gel electrophoresis on 8% SDS−PAGE. Fractions corresponding to the second major [^{32}P]-labeled peak following the dye-front were analyzed by 10% SDS−PAGE and showed a single [^{32}P]-labeled species with an apparent molecular mass of ~26 kDa (5αR-1). TCA precipitation (13.6%) of the labeled fractions resulted in recovery of >70% of the total radioactivity in the protein pellet. Trypsin digestion of the resuspended pellet followed by immobilized-Al^{3+} affinity chromatography indicated that >90% of the radioactivity remained bound to the affinity column. The [^{32}P]-2N$_3$-NADP$^+$-labeled peptide was eluted with potassium phosphate, concentrated, and further purified by reverse-phase (C$_8$) HPLC. Sequence analysis of the purified peptide indicated that it consisted of 11 amino acids with the sequence **N-L-R-K-P-G-E-T-G-Y-K**, corresponding to residues 170−180 of the rat 5αR-1 sequence [Andersson et al. (1989) *J. Biol. Chem. 264,* 16249−16255].

The enzyme 5α-reductase is involved in the NADPH[1] mediated reduction of the $\Delta^{4,5}$ bond of testosterone to 5α-dihydrotestosterone (5α-DHT), the more potent male androgen involved in male sexual differentiation (Mcguire et al., 1960). The enzyme has been implicated in a number of physiological disorders such as benign prostatic hyperplasia (Krieg et al., 1979; Hudson et al., 1983), male pattern baldness (Bingham & Shaw, 1973; Price, 1975), hirsutism among women (Kultenn et al., 1977), and deficiency of this enzyme leads to male pseudohermaphroditism (Imperato-McGinley et al., 1974; Walsh et al., 1974; Moore et al., 1975; Andersson et al., 1991; Thigpen et al., 1992).

The enzyme has been cloned and expressed in mammalian cells (Andersson et al., 1989; Andersson & Russell, 1990; Normington & Russell, 1992; Thigpen et al., 1993), yeast (Ordman et al., 1991), and insect cells (Iehle et al., 1993), and such studies have shown that rats and humans express two different isozymes (designated isozyme-1 and isozyme-2), that can be distinguished by their affinities for steroid substrate, pH optima, and inhibition by certain 4-azasteroids (Normington & Russell, 1992).

Comparison of the cDNA-derived sequences of the rat and human type-1 isozymes has shown that approximately 60% homology exists between the two species, with the similarities being more pronounced at the C-terminal end of the sequence (Andersson et al., 1989; Andersson & Russell, 1990). In addition, hydropathy plots of the two enzymes were almost identical, indicating that they have similar secondary structures. This is reflected in the biochemical similarities between the two isozymes, in that they display similar pH optima, and affinities for physiological steroid substrates. However, the inhibition of the two isozymes by the 4-azasteroid finasteride was markedly different, indicating significant differences in their inhibitor domains.

Thigpen and Russell (1992) have investigated the inhibitor (finasteride) binding domain of the rat and human type-1 isozymes in hybrid 5αR molecules obtained by expression of chimeric cDNAs containing various combinations of rat and human exon sequences. Their studies showed that in the case of the rat enzyme, inhibitor sensitivity was apparently determined by a tetrapeptide (V-S-I-V) in the N-terminal (residues 22−25) region of the 5αR-1 sequence, whereas inhibitor resistance in the human enzyme was conferred by an analogous tetrapeptide in the N-terminal region (A-V-F-A; residues 26−29), and could thus constitute at least a portion of the steroid binding domain. To date, this is the only direct structural information available on the steroid binding domain of the enzyme.

† Supported in part by NIH Grant R01DK41879, by University of Kentucky Medical Center Research Fund, and by The Department of Veterans Affairs.

* To whom correspondence should be addressed at C-410 Research Servive (151), Clinical Addition, VA Medical Center 1101 VA Dr., Lexington, KY 40506. Phone: (606) 233-4511, extension 4592. FAX: (606) 281-4989.

‡ Department of OB/GYN, VA Medical Center.

§ Department of Medicinal Chemistry, University of Kentucky Lucille P. Markey Cancer Center.

|| Department of Biochemistry, University of Kentucky.

⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1995.

[1] Abbreviations: NADPH, nicotinamide adenine dinucleotide phosphate reduced form; NADP$^+$, oxidized nicotinamide adenine dinucleotide phosphate; 2N$_3$-NADP$^+$, 2-azido-NADP$^+$; 5αR-1, 5α-reductase (isozyme-1); DLPC, dilauroylphosphatidylcholine; PS, phosphatidylserine; 5α-DHT, 5α-dihydrotestosterone; PEG-8000, poly(ethylene glycol) (MW 8000); BSA, bovine serum albumin; TFA, trifluoroacetic acid; SR, detergent-solubilized 5αR-1; 3α-ADIOL, 5α-androstane-3α,17β-diol; TCA, trichloroacetic acid.

0006-2960/95/0434-3663$09.00/0

Studies on naturally occurring mutants of the human type-2 5αR obtained from male pseudohermaphrodites (Wigley et al., 1994; Thigpen et al., 1992a) have shown that modification of specific residues leads to decreased affinities for steroid substrate and nucleotide cofactor (Wigley et al., 1994). Modification of N-terminal residues (such as G34R) resulted in decreased affinity for testosterone, consistent with the assignment that the steroid binding domain resides on the N-terminal end of the enzyme molecule. The same study has identified several mutations that occur in the more conserved C-terminal region that resulted in reduced affinities for the pyridine nucleotide cofactor, suggesting that the cofactor binding resides with the C-terminal end of 5αR.

The competitive inhibition of 5α-reductase by 4-methyl-4-azasteroids and the uncompetitive inhibition by 3-androstene-3-carboxylic acids have been extensively characterized (Rasmusson et al., 1984; Liang et al., 1983; 1984; Liang & Heiss, 1981; Levy et al., 1990a). Both types of inhibitors showed an absolute requirement for reduced or oxidized pyridine nucleotide for the formation of the ternary dead-end inhibition complex. In addition, the presence of reduced pyridine nucleotide was also shown to significantly enhance the stability of 5αR in detergent extracts (Levy et al., 1990b). Thus, the interaction between the cofactor and enzyme has both clinical and physiological implications. Despite the extensive kinetic (Campbell & Karavolas, 1989; Cooke & Robaire, 1984; Levy et al., 1990a) and genetic information available, the enzyme has not been purified to homogeneity. Such preparations would permit more extensive physicochemical characterization of the steroid and cofactor binding domains within the 5α-reductase molecule.

We have previously shown that [2′-^{32}P]-2N$_3$-NADP$^+$ is an effective probe of rat liver 5αR-1 (Bhattacharyya et al., 1994). In the current investigation, we have partially purified the (photolabeled) enzyme by preparative SDS gel electrophoresis, digested with trypsin, and purified a [^{32}P]-labeled tryptic peptide by a combination of immobilized-Al^{3+} affinity and reverse-phase high performance liquid chromatography. Sequence data on the purified peptide are presented.

MATERIALS AND METHODS

All reagents used were ACS reagent grade. 1,2,6,7-[^{3}H]-Testosterone ([^{3}H]-T; 90 Ci/mol) used for the assay of 5αR-1 activity in microsomal, detergent-solubilized, and PEG-fractionated preparations was obtained from Amersham Life Sciences, Arlington Heights, IL. NADPH and BSA and iminodiacetic acid−epoxy-activated Sepharose-6B (fast flow) were obtained from Sigma Chemical Co., St. Louis, MO. Modified trypsin was obtained from Promega. [2′-^{32}P]-2N$_3$-NADP$^+$ (3−4 mCi/μmol) was synthesized as described earlier (Bhattacharyya et al., 1994).

Preparation of Microsomes. Microsomes were prepared from the livers of female Sprague-Dawley rats (250−350 g) by homogenizing in 50 mM sodium phosphate, pH 7.0, containing 0.25 M sucrose (buffer A), followed by centrifugation at 10000*g* for 30 min to remove cellular debris, nuclei, and mitochondria (Liang et al., 1983). The supernatant was filtered through four layers of cheesecloth and centrifuged at 100000*g* for 60 min. The pellet was resuspended in buffer A using a Teflon homogenizer, and recentrifuged at 100000*g* for 60 min. The resulting pellet was resuspended in a minimum volume of buffer A and stored at −80 °C. Protein concentrations were determined by the Biuret method (Gornall et al., 1949) using BSA as the standard.

Detergent-Solubilization of Microsomes. Detergent- solubilized preparations containing 5αR-1 activity (SR) were prepared as follows. Microsomes (total protein ~150 mg) were diluted to ~60 mL with buffer A and centrifuged at 100000*g* for 60 min. The pellet was homogenized into half the original volume (~30 mL) of 100 mM sodium citrate, pH 7.5, containing 100 mM KCl, 0.4% Lubrol, 20% glycerol, and 200 μM NADPH (buffer B). The detergent extract was stirred at 4 °C for 30 min and centrifuged at 100000*g* for 60 min. The pellet was discarded and the supernatant stored at −80 °C.

PEG Fractionation of SR. PEG precipitation of SR was carried out by dropwise addition of a cold 50% solution of PEG-8000 (Sigma) such that the final concentration was 6.5% (Ichihara & Tanaka, 1987a, 1991). The mixture was allowed to stir for 30 min, followed by centrifugation at 10000*g* for 30 min (4 °C). The supernatant was discarded, and the pellet containing greater than 85% of the 5αR-1 activity was resuspended in (25% of the original volume of SR) 20 mM sodium citrate, pH 7.5, containing 20 mM KCl, 0.2% Lubrol, 20% glycerol, and 2 μM NADPH (buffer C). UV-photolysis of these fractions with [2′-^{32}P]-2N$_3$-NADP$^+$ was carried out immediately following their preparation.

(A) 5α-Reductase Assay. 5αR-1 activity was assayed by measuring the formation of the [^{3}H]-5α-DHT and [^{3}H]-3α-ADIOL, using an isocratic HPLC system described earlier (Bhattacharyya et al., 1994). Typically, activities of 3−4 nmol min^{-1} mg^{-1} (microsomes), 4−5 nmol min^{-1} mg^{-1} (SR), and 12−16 nmol min^{-1} mg^{-1} (6.5% PEG fraction) were obtained for 5αR-1 at pH 6.5. All incubations were carried out at 37 °C with 5 μM [^{3}H]-T and 200 μM NADPH, respectively.

(B) Photolysis of 5αR-1. Four milliliters of the PEG-fractionated preparation (12−20 mg of total protein) was diluted with 4 mL of 20 mM sodium phosphate (final concentration 1.5−2.5 mg/mL) and preincubated with 5 μM [2′-^{32}P]-2N$_3$-NADP$^+$ (3−4 mCi/μmol) for 15 min at 4 °C, followed by UV-photolysis (254 nm; 4000 μW/cm^2). This was followed by the addition of a second 5 μM aliquot of the probe, preincubation for 15 min, and photolysis for 10 min. The photolyzed sample was reprecipitated with 50% PEG (final concentration 6.5%) and centrifuged at 10000*g* for 30 min. The supernatant was discarded and the pellet washed with 20 mM sodium phosphate. Protein-solubilizing mix (PSM: 125 mM Tris-HCl, pH 6.8, 4% SDS, 1.4 M β-mercaptoethanol, 20% glycerol, and Pyronin-Y dye) was added to solubilize the pellet, and this sample was subjected to preparative gel electrophoresis on 8% SDS−PAGE.

Preparative Gel Electrophoresis. Preparative gel electrophoresis was performed on a BIO-RAD model 491 prep cell. Gel and running buffers were thoroughly degassed prior to preparation of the gel and before each run. The 8% running gel (pH 9.0) had the dimensions 7.2 cm (height) and 3.7 cm (i.d.). A 2 cm 4% stacking gel (pH 6.8) was poured immediately before each run. Samples, typically 1.0−1.5 mL in PSM, were carefully layered on top of the stacker, and electrophoresed at 20 mA constant current (400 V) until the Pyronin-Y dye front entered the main separating gel, at which stage the current was increased to 40 mA. After approximately 4 h, the dye front began eluting from the gel, at which stage the elution buffer (0.1 M NH_4HCO_3, pH 8.0)

Table 1: Yield of ^{32}P-Labeled Protein during Purification

sample	yield (nmol)[a]
5αR peak fractions from preparative 8% SDS−PAGE	1.995
13.6% TCA supernatant	0.53
predigest protein pellet	1.28
peak fractions from immobilized-Al^{3+} affinity column	0.773
peak fractions from reverse-phase HPLC	0.534[b]

[a] Yields based on the specific radioactivity of [2′-^{32}P]-2N_3-$NADP^+$, ~3.35 mCi/μmol. [b] 170 pmol submitted for sequence analysis.

and the UV monitor were turned on. Fractions (1.3−1.5 mL) were collected in 5 mL glass vials, and the A_{254} and radioactive profile were monitored by Cerenkov counting. [^{32}P]-Labeled fractions corresponding to the major radioactive peaks (Fig 1A) were saved, and a portion (typically 100 μL) was separated for analysis by 10% SDS−PAGE and autoradiography.

10% SDS−PAGE Analysis of [^{32}P]-Labeled Fractions. One hundred microliter samples corresponding to the radioactive peak fractions were dried in vacuo to remove excess NH_4HCO_3 and resuspended in 50 μL of distilled water and 50 μL of PSM and vortexed at room temperature. These samples were subjected to 10% SDS−PAGE, followed by Coomassie staining, destaining, and autoradiography (8 h) or by scanning (2 h) on an AMBIS 4000 image acquisition and analysis system.

TCA Precipitation and Trypsin Digestion. The main radioactive peak fractions corresponding to 5αR-1 obtained from two individual preparative gel electrophoresis runs (20 mg each) were pooled (27 and 23.4 mL), precipitated with TCA (13.6% final concentration), allowed to stand for 30 min, and centrifuged at 10000*g* for 30 min. The total radioactivity in the sample and corresponding supernatant following the centrifugation step was determined (Table 1). Each radioactive protein pellet was resuspended in 1.0 mL of 100 mM NH_4HCO_3, pH 7.0, containing 0.05% Triton X-100, and a 20 μL portion was saved. This was followed by the addition of 10 μg of trypsin to each of the 1 mL fractions, followed by incubation at room temperature for 14 h.

(A) Immobilized-Al^{3+} Affinity Chromatography. 1.4 mL of iminodiacetic acid−epoxy-activated Sepharose-6B fast flow was placed in a 15 mL plastic cloumn and washed successively with 2 × 10 mL of distilled water, 15 mL of 50 mM $AlCl_3$ (flow rate 15 mL/h) , 10 mL of water, and 10 mL of 100 mM ammonium acetate, pH 5.9.

The individual digests (1 mL each) were diluted to 10 mL with 100 mM ammonium acetate, pH 5.9, and the pH was adjusted to 5.9 by the addition of 190 μL of glacial acetic acid and combined (total volume 22 mL). This was applied to the immobilized-Al^{3+} affinity column at a flow rate of 6 mL/h, and the eluate was collected in 1.5 mL fractions. The binding of the [^{32}P]-labeled peptides to the affinity column was monitored by checking the radioactivity in the flow-through and by a Geiger counter. Typically, greater than 90% of the radioactive peptide remains bound to the upper third of the column and few losses were encountered. The column was washed with 15 mL of buffer D, 5 mL of buffer D + 0.5 M NaCl, and 5 mL of buffer D. The peptides were eluted by addition of 1 mL of 100 mM ammonium acetate, pH 8.0, containing 10 mM sodium phosphate (buffer E) followed by incubation for 10 min and subsequent elution, during which 0.5 mL fractions were collected. This step was repeated until a majority of the bound radioactivity was eluted. The peak fractions were saved and further purified by reverse-phase HPLC. The total yield of peptide obtained was determined by liquid scintillation counting.

(B) Reverse-Phase High-Performance Liquid Chromatography. The peak fractions obtained from the immobilized-Al^{3+} affinity column (39−43, Figure 3) were combined to give a total volume 1.4 mL. To this was added 0.3 mL of 0.1% TFA and the entire mixture subjected to reverse-phase HPLC, using an Aquapore RP-300 C_8 column (Brownlee Labs). Optical density and spectral data were obtained with an LKB HPLC system connected in series to a diode array spectral detector. The column was developed at a flow rate of 30 mL/h with a 70 min gradient of solvent F (0.1% trifluoroacetic acid) to solvent G (70% acetonitrile in 0.1% TFA). The column was developed as follows: solvent F, 100% 0−10 min with a linear increase in solvent G from 0% at 10 min to 100% at 70 min. Fractions (0.5 mL) were collected at 1.0 min intervals, and the radioactivity was monitored. The main radioactive peak eluted at 32 min. No other radioactive species could be detected, although a small amount of radioactivity coeluted with the solvent front. The fraction containing most of the radioactivity was concentrated to ~150 μL and was submitted for sequence analysis at The University of Kentucky Macromolecular Structure Facility, using an Applied Biosystems 477A pulse liquid protein sequencer with on-line identification of phenylthiohydantoin derivatives.

RESULTS

In an earlier publication, we described the photolabeling of microsomal, detergent-solubilized, and PEG-fractionated preparations containing 5αR-1 activity by [2′-^{32}P]-2N_3-$NADP^+$ (Bhattacharyya et al., 1994). In this study, we demonstrated that the probe was a competitive inhibitor of NADPH (K_i ~ 17 μM) and furthermore reported the saturation labeling of 5αR-1 by the probe which was maximal at ~10 μM and obtained an apparent K_d of ~2.0 μM. The time-dependent inactivation (measured as a function of increasing photolysis time) and subsequent incorporation of radioactivity into the enzyme were also investigated in the presence of the photoprobe, and a $t_{1/2}$ of ~1.0 min for the photolabeling and subsequent inactivation of 5αR-1 by [2′-^{32}P]-2N_3-$NADP^+$ was obtained. UV-photolysis of the enzyme in the absence of the probe did not significantly reduce enzyme activity, indicating that it remains stable under the conditions of photolysis employed in these experiments. The (26 kDa) 5αR-1 protein band was specifically protected from labeling by the inclusion of excess $NADP^+$, and enzyme activity was preserved, whereas excess NAD^+ failed to protect the enzyme from the probe and subsequent loss of enzyme activity was observed.

Figure 1A shows a typical [^{32}P] elution profile obtained upon preparative gel electrophoresis of [^{32}P]-labeled PEG-fractionated preparations (12−20 mg). Two radioactive peaks are observed, the first of which coelutes with the Pyronin-Y dye front; 10% SDS−PAGE analysis followed by autoradiography showed that this peak consists mainly of unbound probe (and photoproducts) and some minor photolabeled protein bands (Figure 1B) in the 12−20 kDa range. Fractions from the second radioactive peak were analyzed by 10% SDS−PAGE, by autoradiography, and by

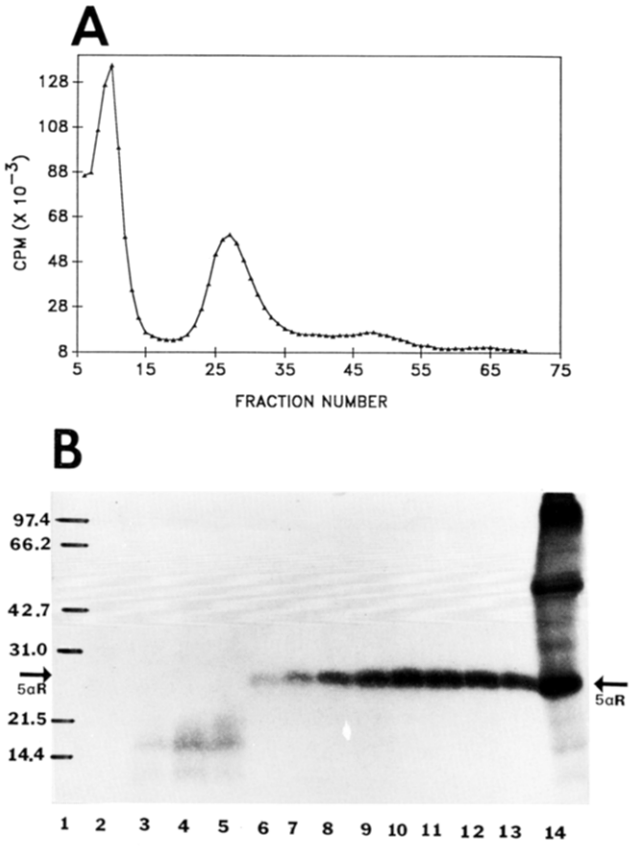

FIGURE 1: (A) Radioactive profile corresponding to the elution of [^{32}P]-labeled proteins obtained from preparative 8% SDS−PAGE (Bio-Rad Model 491 Prep Cell). 6.5% PEG-fractionated preparations (12−20 mg) were photolyzed with [2′-^{32}P]-2N$_3$-NADP$^+$ as described under Materials and Methods, and electrophoresed at constant current for ~4 h. 1.3−1.5 mL fractions were collected in glass vials, and the radioactive profile was determined by Cerenkov counting in a gamma counter. Radioactive peaks were further analyzed by 10% SDS−PAGE and autoradiography. (B) Autoradiogram corresponding to the [^{32}P]-labeled species obtained from 10% SDS−PAGE analysis of radioactive peaks from (A). 100 μL samples of each fraction were dried in vacuo, resusupended in 50 μL of PSM and 50 μL of deionized water, and electrophoresed as described under Materials and Methods. The gel was run as follows: lane 1, 10 μg of protein standards; lane 2, fraction 8; lane 3, fraction 12; lane 4, fraction 14; lane 5, fraction 16; lane 6, fraction 18; lane 7, fraction 22; lane 8, fraction 24; lane 9, fraction 26; lane 10, fraction 28; lane 11, fraction 31; lane 12, fraction 32; lane 13, fraction 33; lane 14, 15 μL of 6.5% PEG fraction. The arrow indicates the position of the radioactive band corresponding to 5αR-1. Autoradiography was for 8 h.

an AMBIS 4000 image acquisition and analysis sytem, and showed that these correspond to a single [^{32}P]-labeled band with an apparent molecular mass of 26 kDa (5αR-1).

Two separate 20 mg fractions were photolyzed twice with 5 μM [2′-^{32}P]-2N$_3$-NADP$^+$, and electrophoresed separately on 8% preparative SDS−PAGE. The pooled peak fractions from each individual run containing [^{32}P]-labeled 5αR-1 were TCA-precipitated, and the resuspended pellet was subject to trypsin digestion. The predigest and postdigest samples were analyzed by 10% SDS−PAGE and by autoradiography, and essentially showed that trypsin treatment resulted in complete digestion of the 26 kDa protein as evidenced by a lack of Coomassie-stained protein in this fraction (Figure 2A). Coomassie staining of the undigested fraction showed the presence of at least four protein bands with apparent molecular masses of 36, 30, 26, and 24 kDa, of which the 26 kDa protein band appears to be the predominant species

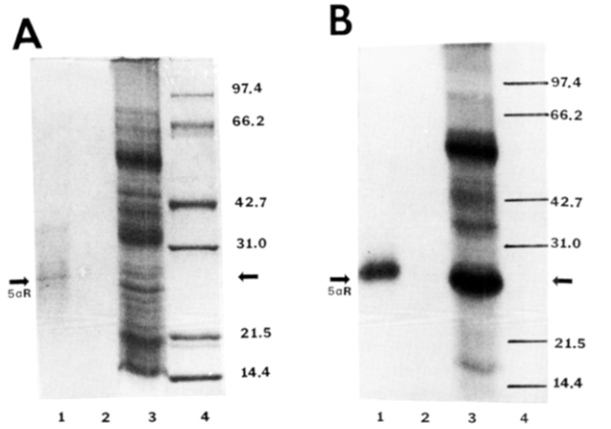

FIGURE 2: (A) Coomassie-stained gel corresponding to an aliquot of undigested and trypsin-digested 5αR-1 fractions obtained from a total of 40 mg of 6.5% PEG fraction after preparative gel electrophoresis. The main 5αR-1 fractions were pooled and TCA-precipitated, and the resuspended pellet was digested with trypsin as described under Materials and Methods. The undigested sample and the (14 h) trypsin digest were electrophoresed on 10% SDS−PAGE. The gel was run as follows: lane 1, combined 5αR-1 predigest, 100 μL; lane 2, trypsin-digested fractions, 100 μL; lane 3, 15 μL of 6.5% PEG fraction; lane 4, 10 μg of protein standards. (B) Autoradiogram corresponding to the combined undigested and trypsin-digested [^{32}P]-labeled 5αR-1 fractions from preparative gel electrophoresis. The position of [^{32}P]-labeled 5αR-1 is indicated by the arrow. Protein standards are indicated by the solid lines. Individual lanes are identical to those described in (A). Autoradiography was for 8 h.

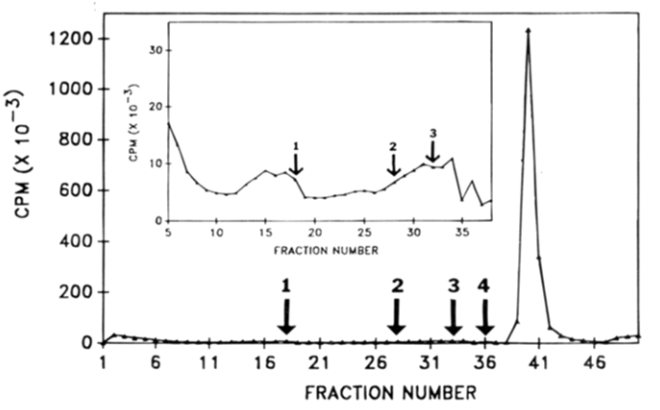

FIGURE 3: (A) Radioactivity profile of tryptic peptides eluting from the immobilized-Al^{3+} affinity column. The tryptic digests from the combined TCA precipitate obtained from two individual preparative gel electrophoresis experiments (20 mg/gel) were loaded onto a 1.4 mL Al^{3+}-chelate column as described under Materials and Methods. Peptide loading, washing, and elution are indicated by the arrows. The inset shows the radioactive profile during the wash steps. Fractions 1−17, column loading; 1, first wash with 10 mL of buffer D, fractions 18−27; 2, second wash with buffer D + 0.5 M NaCl, fractions 28−31; 3, third wash with 5 mL of buffer D, fractions 32−35; 4, peptide elution with buffer E, fractions 36−50. The main peak fractions (39−43) were pooled and further purified by reverse-phase HPLC.

(based on the intensity of staining). Autoradiography showed the presence of a single 26 kDa radiolabeled species in the undigested fraction, which is noticeably absent in the trypsin digest.

The digested fractions were diluted as indicated under Materials and Methods and chromatographed on an immobilized-Al^{3+} affinity column as described earlier. Figure 3 shows the elution profile of the [^{32}P]-labeled peptide fraction. It is clear that only very small losses in bound radioactivity are encountered during the wash steps (inset,

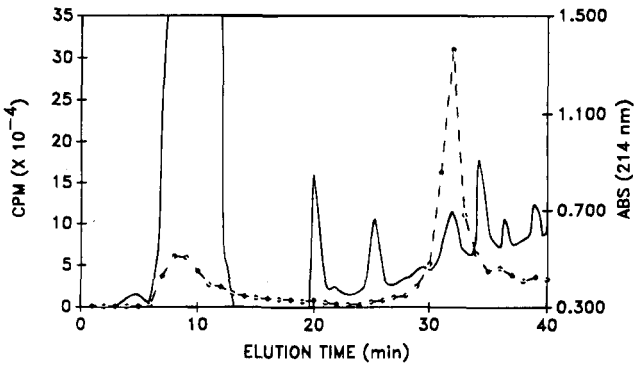

FIGURE 4: Radioactivity and UV (214 nm) absorbance reverse-phase HPLC profile of peak fractions from immobilized-Al^{3+} affinity chromatography. Combined 5αR-1-containing fractions were digested with trypsin and subject to Al^{3+}-chelate chromatography as described under Materials and Methods. The peptides in the corresponding radioactive fractions from the immobilized metal-affinity column were seperated by reverse-phase HPLC using a C_8 column with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid as described under Materials and Methods. (—) A_{214nm} profile; (●- - -●) radioactivity profile. The main radioactive peak fraction (elution time = 32 min) was concentrated to 150 μL and submitted for sequence analysis.

Figure 3). Once the radioactivity reaches base-line levels, elution of the bound peptide is achieved by increasing the pH of the elution buffer to 8.0 and inclusion of 10 mM PO_4^{3-}. Elution of the peptide was achieved in a narrow peak and in a minimum volume.

Sample preparation for HPLC was performed as indicated under Materials and Methods. Elution was monitored by following the UV absorbance characteristics at 214 nm, and by monitoring the radioactive profile. Figure 4 shows the elution profile obtained from such an experiment. Only one major radioactive peak is observed at 32 min and corresponds to a single peptide (214 nm) peak. Five other peptides were also resolved, none of which showed the corresponding radioactivity. These peptides are presumably retained by the affinity support by nonspecific interactions. An examination of the HPLC profile of the flow-through fractions from the immobilized-Al^{3+} affinity column showed a complex mixture of peptides (data not shown). A small amount of radioactivity coelutes with the solvent front, and is probably a result of partial hydrolysis of the bound probe from the peptide that occurs during these procedures. The stability of the covalent linkage is demonstrated by the percentage of radioactivity recovered in the the main peptide peak (75%) versus losses in the solvent front (25%). The main peak fraction (32 min) containing the highest radioactivity was submitted for sequence analysis, and yielded the following sequence: **Asn-Leu-X-Lys-Pro-Gly-Glu-Thr-Gly-Tyr-Lys**, corresponding to residues 170−180 of the 5αR-1 sequence. Alignment of the peptide sequence to the published rat 5αR-1 sequence shows that the missing amino acid (denoted by X) corresponds to ^{172}Arg and appears to be the residue that may be covalently modified by the photoprobe.

DISCUSSION

Identification of the peptide domains that influence the binding of substrate and cofactor and subsequent catalysis is an important step in understanding structure−function relationships in virtually any enzyme system. In the case of steroid 5α-reductase, limited data exist on residues directly involved in binding the substrate/inhibitors and pyridine nucleotide cofactor.

Although 5αR has been detergent-solubilized and partially purified from different tissue sources (Sargent & Habib, 1991; Levy et al., 1990a; Ichihara & Tanaka, 1987a), conventional methods for the purification of the rat liver enzyme such as ion-exchange (DEAE-cellulose), hydrophobic (phenyl/octyl-Sepharose), and affinity chromatography ($NADP^+$-linked agarose) (Golf & Graef, 1978; Levy et al., 1990a) were all met with limited success. The biggest problem encountered in these procedures was the loss of enzyme activity upon binding of crude preparations to these supports. Several resesarch groups have shown that inclusion of phospholipids (DLPC, PS) in crude preparations of 5αR resulted in partial recovery and stabilization of activity (Golf & Graef, 1978; Ichihara & Tanaka, 1987a,b, 1991; Sargent & Habib, 1991). Our own attempts at purifying the enzyme from PEG-fractionated preparations were met with limited succcess. Attempts to purify the enzyme using affinity supports such as 17β-carboxamido-4-methyl-4-aza-5α-androstanaminooctyl-Sepharose and 2′-5′-ADP−agarose (Sigma) were all unsuccessful in that either no binding (4MA-affinity) or virtually complete loss of enzyme activity (nucleotide-affinity) was encountered. Inclusion of phospholipids (DLPC + PS) did not significantly alter the binding characteristics of the enzyme to these supports.

Preparative gel electrophoresis is a technique that has been used with a fair amount of success for the purification of both native and denatured proteins in quantities that permit their physicochemical characterization (Hager & Wright, 1992; Sanchez et al., 1992; Hughes et al., 1990). We previously demonstrated that [2′-^{32}P]-2N_3-$NADP^+$ covalently labels 5αR-1 in PEG-fractionated preparations, and appears to form an acid-stable chemical bond with the enzyme as evidenced by its stability to staining and destaining procedures employed for visualization of the enzyme on SDS−PAGE. The stability of this linkage permitted us to partially purify the [^{32}P]-labeled (5αR-1) fraction under denaturing conditions. Although other Coomassie-stained bands were observed in these fractions, the major protein species appeared to be 5αR-1. Digestion of these fractions leads to a mixture of both 5αR-1 and other contaminating peptides. The immobilized metal ion (Al^{3+}) affinity column employed has a high affinity for peptides covalently linked to nucleotide moieties by coordinating to the negatively charged phosphate groups present, and has been sucessfully employed in the purification of photolabeled peptides in several systems (Shoemaker & Haley, 1993; Salvucci et al., 1992; Chavan et al., 1992). Although use of these supports significantly improves the purity of the photolabeled peptides, it also shows some preference for acidic peptides. The presence of such nonspecifically bound peptides is apparent from analysis of the chelate-derived peptides by reverse-phase HPLC (Figure 4). However, the HPLC step results in the final purification of the peptide to homogeneity, as evidenced by the lack of significant background during sequencing procedures.

One of the most frequent problems encountered during the reverse-phase HPLC purification of azidopurine-labeled peptides in the past has been the lability of the radioactive label during this step. Although the reason for this is unclear, it is most likely that the stability of the covalent linkage is determined by the nature of the amino acid residue to which

Table 2: Amino Acid Sequence Analysis of the Photolabeled Tryptic Peptide

cycle no.	amino acid (pmol)	cycle no.	amino acid (pmol)
1	^{170}Asn (140)	7	Glu (89)
2	Leu (107)	8	Thr (92)
3[a]	(Arg)	9	Gly (71)
4	Lys (117)	10	Tyr (64)
5	Pro (114)	11	^{180}Lys (50)
6	Gly (98)		

[a] Arg expected from the primary sequence, but was not detected.

it becomes attached. As much as 75% of the bound label can be lost during the final purification step (Jayaram & Haley, 1994; Salvucci et al., 1992). The data presented here on 5αR-1 are perhaps the first instance where this is not a serious problem, and therefore represents one of the most stable linkages investigated to date by this research group, and thus permits tentative assignment of the residue covalently modified by the probe. Previous studies on the identification of nucleotide binding domains in proteins have been accomplished using purified proteins. In this study, the protein of interest was photolabeled in relatively crude preparations (PEG fraction) and partially purified, and the nucleotide binding domain was subsequently identified. This methodology may therefore be applicable to other systems that are difficult to purify to homogeneity.

Sequence analysis of the HPLC-purified peptide indicated that it consists of 11 amino acid residues of the following sequence:

-N-L-X-K-P-G-E-T-G-Y-K-

These correspond to residues 170−180 of the rat 5αR-1 sequence (Andersson et al., 1989, Andersson & Russell, 1990), and most likely correspond to a portion of the $NADP^+$ (adenine) binding domain of 5αR-1 (Table 2). The only amino acid not identified during the sequencing procedure corresponds to ^{172}Arg and appears to be the residue modified by the probe. The tryptic peptide appears to be situated in a glycine-rich region of the polypeptide (residues 175−185), but bears little homology to the commonly found Gly-X-Gly-X-X-Gly/Ala sequence commonly associated with NAD^+/$NADP^+$ binding enzymes (Segal et al., 1992; Baker et al., 1992). Although the overall sequence of the rat 5αR-1 sequence shows little sequence identity with other proteins, sufficient homology exists between the human and rat isozymes to draw the following conclusions.

(1) The peptide is situated in a highly conserved region of the 5αR sequence, and this region (residues 160−190 for rat type-1) is virtually identical (~97% homologous) to that of the human type-1 isozyme (residues 164−194) except that ^{176}Glu is substituted by ^{180}Asp in the human type-1 sequence (Normington & Russell, 1992; Andersson & Russell, 1990). Comparison of the rat and human type-2 isozymes to the type-1 sequences once again shows a fair amount of homology in the 30-residue peptide region shown below (~77% for both rat and human type-2 vs the rat type-1) [partial sequences shown are from Normington and Russell (1992)]:

^{160}INIHSDHILRNLRKP**GETGYK**IPRGGLFEYV^{190}
(rat 5αR-1)

^{164}INIHSDHILRNLRKP**GDTGYK**IPRGGLFEYV^{194}
(human 5αR-1)

^{159}INIHSDYILRQLRKP**GEISYR**IPQGGLFTYV^{189}
(human 5αR-2)

^{159}INIHSDYTLRQLRKP**GEVIYR**IPRGGLFTYV^{189}
(rat 5αR-2)

(2) The hydropathy plot (Andersson et al., 1989) indicates that the peptide is located in a solvent-exposed portion of the 5αR-1 sequence and can thus interact directly with the hydrophilic NADP(H) molecule.

(3) The type-1 5α-reductases therefore appear to have a unique nucleotide binding sequence that can be tentatively assigned as:

Gly-X-X-Gly-X-X-X-X-X-Gly-Gly

whereas in the type-2 binding domain three out of the four Gly residues are conserved and the second glycine residue is replaced either by Ser (human type-2) or by Ile (rat type-2).

(4) The similarities between the putative binding domains is clear upon closer examination of the residues denoted by X. In both cases, an acidic residue immediately follows the first glycine, that could possibly be involved in hydrogen bonding to the the ribose moiety, or to the ring nitrogens of the adenine ring. Second, all appear to have a conserved aromatic residue which may be involved in aromatic and H-bonding interactions with the adenine ring of $NADP^+$.

Wigley et al. (1994) in a natural mutagenesis study of the human type-2 isozyme have identified several mutations in the type-2 gene that lead to 5α-reductase deficiency among a certain population of male pseudohermaphrodites. Ten of these missense mutations out of a total of 22 were shown to alter either the binding affinity of the steroid substrate or that of the pyridine nucleotide. Eight of these mutations resulted in an altered binding affinity for NADPH, while the remaining two mutations resulted in decreased affinity for testosterone. The remaining 12 resulted in the inactivation of the enzyme.

The mutations R171S and P181L and G183S (Wigley et al., 1994) in human 5αR-2 all lie within the proposed adenine binding domain of the enzyme. Sequence alignments show that these residues are highly conserved in all four 5αR isozymes, and in fact the corresponding residue R172 in the rat 5αR-1 sequence is shown to be covalently modified by the probe. These observations lend further support in favor of the proposed $NADP^+$ binding domain. Other mutations such as R145W, N193S, G196S, and R246Q(W) that affect NADPH binding could also be involved in adenine binding or may interfere with binding of the nicotinamide ring or interaction with the ribose hydroxyl groups or the 2′-phosphate. Further studies are necessary to examine these possibilities.

The data presented here describe a relatively straightforward three-step purification of a photolabeled tryptic peptide corresponding to the NADP(H) binding domain of 5αR-1. Future studies on this isozyme will be carried out to identify the peptide domains and specific residues that influence the binding of the nicotinamide ring of NADP(H) as well as the

steroid substrate and inhibitor molecules. Such studies are currently underway in this laboratory.

REFERENCES

Andersson, S., & Russell, D. W. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3640−3644.

Andersson, S., Bishop, R. W., & Russell, D. W. (1989) *J. Biol. Chem. 264*, 16249−16255.

Andersson, S., Berman, D. M., Jenkins, E. P., & Russell, D. W. (1991) *Nature 354*, 159−161.

Baker, P. J., Britton, L. K., Rice, D. W., Rob, A., & Stillman, T. J. (1992) *J. Mol. Biol. 228*, 662−671.

Bhattacharyya, A. K., Chavan, A. J., Shuffett, M., Haley, B. E., & Collins, D. C. (1994) *Steroids 59*, 634−641.

Bingham, K. D., & Shaw, D. A. (1973) *J. Endocrinol. 57*, 111−121.

Campbell, J. S., & Karavolas H. J. (1989) *J. Steroid Biochem. 32*, 283−289.

Cooke, G. M., & Robaire, B. (1984) *J. Steroid Biochem. 20*, 1279−1284.

Cooke, G. M., & Robaire, B. (1987) *J. Steroid Biochem. 26*, 581−588.

Golf, S. W., & Graef, V. (1978) *J. Steroid Biochem. 9*, 1087−1092.

Hager, K. M., & Wright, E. M. (1992) Bio-Rad US/EG Bulletin 1685.

Houston, B., Chisholm, G. D., & Habib, F. K. (1985) *J. Steroid Biochem. 22*, 461−467.

Houston, B., Chisholm, G. D., & Habib, F. K. (1987) *Steroids 49*, 355−369.

Hudson, R. W., Moffitt, P. M., & Owens, W. A. (1983) *Can. J. Biochem. Cell Biol. 61*, 750−755.

Hughes, G. J., Frutiger, S., Paquet, N., & Jaton, J. C. (1990) *Biochem. J. 271*, 641−647.

Ichihara, K., & Tanaka, C. (1987a) *Biochem. Int. 15*, 1005−1011.

Ichihara, K., & Tanaka, C. (1987b) *Biochem. Biophys. Res. Commun. 149*, 482−487.

Ichihara, K., & Tanaka, C. (1991) *Lipids 26*, 531−535.

Iehle, C., Delos, S., Filhol, O., & Martin, P. (1993) *J. Steroid Biochem. Mol. Biol. 46*, 177−182.

Imperato-McGinley, J., Guerrero, J., Gautier, T., & Petersen, R. E. (1974) *Science 186*, 1213−1215.

Imperato-McGinley, J., & Petersen, R. E. (1976) *Am. J. Med. 61*, 251−272.

Jayaram, B., & Haley, B. E. (1994) *J. Biol. Chem. 269*, 3233−3242.

Jenkins, E. P., Andersson, S., Imperato-McGinley, J., Wilson, J. D., & Russell, D. W. (1992) *J. Clin. Invest. 89*, 293−300.

Krieg, M., Bartsch, W., Janssen, W., & Voigt, K. D. (1979) *J. Steroid Biochem. 11*, 615−624.

Levy, M. A., Brandt, M., & Greway, A. T. (1990a) *Biochemistry 29*, 2808−2815.

Levy, M. A., Brandt, M., Heys, J. R., Holt, D. A., & Metcalf, B. W. (1990b) *Biochemistry 29*, 2815−2824.

Liang, T., & Heiss, C. E. (1981) *J. Biol. Chem. 256*, 7998−8005.

Liang, T., Heiss, C. E., Ostrove, S., Rasmusson, G. H., & Cheung, A. (1983) *Endrocinology 112*, 1460−1468.

Liang, T., Heiss, C. E., Cheung, A. H., Reynolds, G. F., & Rasmusson, G. H. (1984) *J. Biol. Chem. 259*, 734−739.

McGuire, J. S., Hollis, V. W., & Tomkins, G. M. (1960) *J. Biol. Chem. 235*, 3112−3117.

Moore, R. J., Griffin, J. E., & Wilson, J. D. (1975) *J. Biol. Chem. 250*, 7168−7172.

Normington, K., & Russell, D. W. (1992) *J. Biol. Chem. 267*, 19548−19554.

Ordman, A. B., Meyhack, B., & Hanspeter, N. (1991) *J. Steroid Biochem. Mol. Biol. 39*, 487−492.

Price, V. H. (1975) *Arch. Dermatol. 111*, 1496−1502.

Rasmusson G. H., Reynolds G. F., Utne T., Jobson R. B., Primka R. L., Berman C., & Brooks J. R. (1984) J. Med. Chem. *27*, 1690 - 1701.

Rasmusson, G. H., Reynolds, G. F., Steinberg, N. G., Walton, E., Patel, G. F., Liang, T., Casscieri, M. A., Cheung, A. H., Brooks, J. R., & Berman, C. (1986) *J. Med. Chem. 29*, 2298−2315.

Salvucci, M. E., Chavan, A. J., & Haley, B. E. (1992) *Biochemistry 31*, 4479−4487.

Sanchez, J. C., Paquet, N., Hughes, G., and Hochstrasser, D. (1992) Bio-Rad US/EG Bulletin 1744.

Sansone, G., & Reisner, R. M. (1971) *J. Invest. Dermatol. 56*, 366−372.

Sargent, N. S. E., & Habib, F. K. (1991) *J. Steroid Biochem. Mol. Biol. 38*, 73−77.

Segal, A. W., West, I., Wientjes, F., Nugent, J., Chavan, A. J., Haley, B. E., Garcia, R. C., Rosen, H., & Scrace, G. (1992) *Biochem. J. 284*, 781−788.

Shoemaker, M. T., & Haley, B. E. (1993) *Biochemistry 32*, 1883−1890.

Thigpen, A. E., & Russell, D. W. (1992) *J. Biol. Chem. 267*, 8577−8583.

Thigpen, A. E., Davis, D. L., Milatovich, A., Mendonca, B., Imperato-McGinley, J., Griffin, J. E., Francke, U., Wilson, J. D., & Russell, D. W. (1992) *J. Clin. Invest. 90*, 799−809.

Thigpen, A. E., Cala, K. M., & Russell, D. W. (1993) *J. Biol. Chem. 268*, 17404−17412.

Walsh, P. C., Madden, J. D., Herrod, M. J., Goldstein, J. L., MacDonald, P. C., & Wilson, J. D. (1974) *N. Engl. J. Med. 291*, 944−949.

Wigley, C. W., Prihoda, J. S., Mowszowicz, I., Mendonca, B. B., New, M. I., Wilson, J. D., & Russell, D. W. (1994) *Biochemistry 33*, 1265−1270.

BI9421376