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Androgen-binding Protein

PURIFICATION FROM RAT EPIDIDYMIS, CHARACTERIZATION, AND IMMUNOCYTOCHEMICAL LOCALIZATION*

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Androgen-binding protein (ABP) was purified from caput epididymis of the rat by sequential chromatography on DEAE-Sepharose, hydroxylapatite, dihydrotestosterone-17β-hemisuccinyl-1,6-diaminohexane-Sepharose, and Sephadex G-150. The final product migrated as a single band corresponding to a peak of protein-bound [^{3}H]dihydrotestosterone on polyacrylamide gel electrophoresis. A molecular weight of 100,000 was estimated by sedimentation equilibrium. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis, subunits of M_r = 47,000 and 41,000 were observed. Amino acid analysis indicated ABP to be rich in leucine while nonpolar aminoacids totaled only 51%. Its carbohydrate content is 25%. Antibodies to purified ABP were raised in a rabbit and evaluated by immunodiffusion, immunoelectrophoresis, binding inhibition, radioimmunoassay, and immunocytochemistry. Immunoperoxidase staining localized ABP in the basal and adluminal regions of seminiferous tubules of rat testis and in secretory granules of cultured Sertoli cells. In principal cells of caput epididymis, ABP is concentrated in the supranuclear region known to contain morphological specializations for absorption. These immunocytochemical results confirm that ABP synthesized and secreted by Sertoli cells in the testis is transported to the epididymal duct via testicular fluid and is taken up by epithelial cells of the proximal segments.

The discovery of androgen-binding protein in epididymis and testicular fluid (1–3) and its subsequent identification as a specific Sertoli cell secretory protein (4–6) has provided a valuable marker for studies on the hormonal regulation of Sertoli cell function (7–11). Until recently, however, ABP[1] was identified solely by its androgen-binding activity.

We have purified ABP from rat epididymis.[2] The purified protein has been characterized and antiserum has been raised in rabbits. Immunocytochemical studies show specific staining of Sertoli cells, the site of ABP production, and of epithelial cells in the caput epididymis, where ABP is absorbed from the testicular fluid.

METHODS

Chemical Analysis—Protein (100 μg) was hydrolyzed in 6 N HCl in evacuated, sealed tubes for 24 h. Hydrolysate was analyzed in a Bio-Cal BC 200 automatic amino acid analyzer equipped with an Autolab integrator. Cystine was also determined as cysteic acid in an independent run following performic acid oxidation. Tryptophan was not analyzed. Carbohydrate composition was determined by gas-liquid chromatography (12).

Analytical Ultracentrifugation—Equilibrium sedimentation was carried out at 20 °C for 22 h at 15,000 rpm in an MSE Centriscan 75 centrifuge equipped with UV scanning optics. Sample consisted of a 0.04% solution of ABP in 50 mM Tris-HCl buffer, pH 7.4, containing 1M KCl, 10% glycerol, and 0.01% sodium azide.

SDS-Polyacrylamide Gel Electrophoresis—Cylindrical gels (5 × 60 mm) were prepared according to Laemmli (13) with the exception that gels contained 4 M urea and 1 mM EDTA. Stacking and running gels were 3.6 and 8% acrylamide, respectively. Electrophoresis buffer contained 0.096 M glycine, 0.025 M Tris, and 0.1% SDS. Protein samples were suspended in 1% sodium dodecyl sulfate, 2.5 mM EDTA, 2.75 M urea, 50 mM Tris-HCl, pH 6.7, 3% β-mercaptoethanol, 0.05% bromphenol blue and solubilized by heating 10 min at 60 °C. Samples were electrophoresed for 2 h at 1.5 mA/tube. Protein was stained with 0.25% Coomassie blue in 50% methanol, 10% acetic acid and destained with 20% methanol, 10% acetic acid.

Preparation of Antiserum—Lyophilized antigen (100 μg) was dissolved in 2 ml of saline (0.9% NaCl solution) and emulsified with an equal volume of Freund's complete adjuvant and 10 mg of *mycobacterium butyricum*. A New Zealand white rabbit was immunized as described by Vaitukaitis *et al.* (14). Booster injections of antigen (100 μg) were given 2 and 4 months following the primary immunization, and the rabbit was bled 10 days following each booster.

Evaluation of Antiserum—ABP antibodies were detected by double immunodiffusion (15) against purified ABP. Specificity was further examined by crossed immunoelectrophoresis according to Laurell (16) and by inhibition of ABP binding of [^{3}H]dihydrotestosterone on polyacrylamide gel electrophoresis (17).

RESULTS

ABP was purified by column chromatography on DEAE-Sepharose, hydroxylapatite, [^{3}H]dihydrotestosterone hemisuccinate coupled to 1,6-diaminohexane Sepharose, and Sephadex G-150 (Fig. 1). Purified ABP formed a single band (*RF* = 0.5 relative to bromphenol blue) on polyacrylamide gel electrophoresis and corresponded to a peak of bound radio-

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[1] The abbreviations and trivial names used are: ABP, androgen-binding protein; SDS, sodium dodecyl sulfate; dihydrotestosterone, 17 β-hydroxy-5 α-androstane-3-one.

[2] Portions of this paper (including some "Methods," Figs. 1–5, Table I, and additional references) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20014. Request Document No. 80M-1811, cite author(s), and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

activity (Fig. 2). Equilibrium centrifugation of intact ABP indicated a single component system. Observed data fit well into the Lamm equation (Fig. 3), yielding a molecular weight estimate of 100,000 using a partial specific volume of 0.71 cm^3/g as determined from the amino acid composition. The homogeneity of ABP was also examined by electrophoresis in a 10% SDS-polyacrylamide gel (Fig. 4). ABP migrated as two major bands corresponding to molecular weights of 41,000 and 47,000 in a ratio of approximately 1:3, respectively.

The chemical composition of ABP is shown in Table I and some of its physicochemical properties are listed in Table II. ABP is exceptionally rich in leucine, even though the content of nonpolar amino acids (51%) is not extraordinary. Its hydrophobicity is average whereas fractional charge is relatively high. The carbohydrate content of ABP is unique in that it is high in glucose. The amount of *N*-acetylglucosamine recovered in the amino acid analyzer (12.2 nmol) was in acceptable agreement with the amount obtained by carbohydrate analysis (14.9 nmol).

Immunization of a rabbit with ABP raised precipitating antibodies, the highest titer being reached following the second booster. Antiserum was judged to be monospecific by immunodiffusion (not shown) and crossed immunoelectrophoresis (Fig. 5) against purified ABP. Single precipitin lines were observed with both methods. No precipitation was observed with preimmunization serum from the same rabbit.

Further evidence that the immune serum contained antibodies against ABP was obtained by quantitation of [^{3}H]-dihydrotestosterone binding during polyacrylamide gel electrophoresis (Fig. 6). Incubation of epididymis cytosol with

FIG. 1. **Procedure for purification of ABP from rat epididymis.**

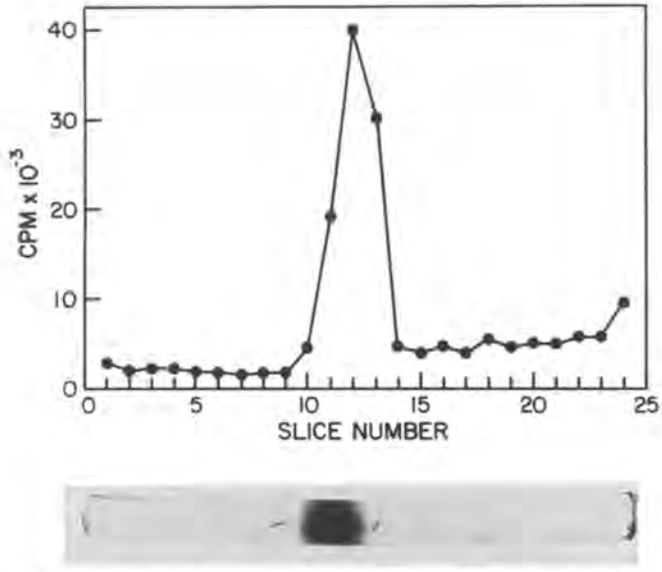

FIG. 2. **Electrophoresis of purified ABP, 15 μg, in a 6.5% polyacrylamide gel (5 × 60 mm) containing 2 nM [^{3}H]dihydrotestosterone (18).** Electrophoresis was performed in Tris/glycine buffer, pH 8.9, at 2 to 4 °C, 2 mA/tube, and continued until the tracking dye, bromphenol blue, reached the end of the gel. The gel was sliced in half lengthwise and corresponding segments of the two halves were marked with wires. One half was sliced into 2.3-mm cross-sectional segments which were eluted and counted in 5 ml of toluene-based scintillator. The other half was stained with Coomassie brilliant blue G.

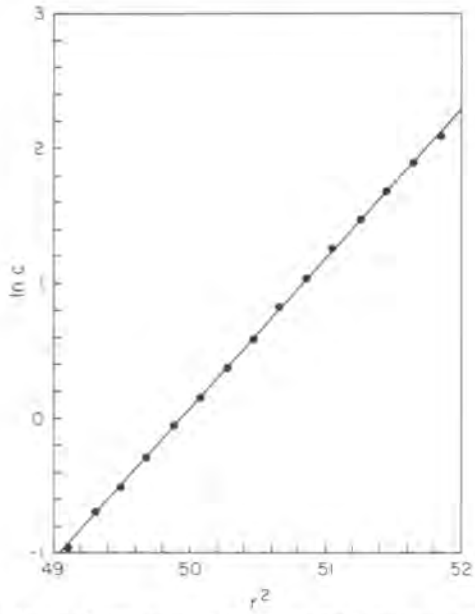

FIG. 3. **Data from equilibrium centrifugation of purified ABP plotted according to the Lamm equation (19), where *c* is the concentration of protein and *r* is the distance from center of rotation.**

FIG. 4. **Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified ABP (20 μg).** Protein was suspended in 1% sodium dodecyl sulfate, 2.5 mM EDTA, 2.75 M urea, 50 mM Tris-HCl, pH 6.7, 3% 2-mercaptoethanol, 0.05% bromophenol blue, and heated for 10 min at 60 °C. Cylindrical stacking and running gels (5 × 60 mm) contained 3.6 and 8% acrylamide, respectively. Electrophoresis was at 4 °C, 1.5 mA/tube for 2 h. Protein was stained with Coomassie blue. Molecular weight markers were bovine serum albumin (M_r = 68,000), ovalbumin (M_r = 43,000) and chymotrypsinogen-α (M_r = 23,000).

TABLE I

Chemical composition of rat androgen-binding protein

Residue	g/100 g peptide	Moles/mol protein[a]
Aspartic acid	15.25	86
Threonine	5.37	34
Serine	8.32	59
Glutamic acid	10.28	52
Proline	5.88	38
Glycine	5.00	50
Alanine	3.85	32
Half-cystine	1.22	8
Valine	4.48	29
Methionine	1.37	7
Isoleucine	2.06	12
Leucine	19.00	109
Tyrosine	0.55	2
Phenylalanine	4.19	19
Histidine	3.49	17
Lysine	3.89	20
Arginine	5.82	25
	g/100 g protein	
Mannose	3.29	18
Glucose	15.41	86
N-Acetylglucosamine	3.25	15
N-Acetylneuraminic acid	3.05	10

[a] Adjusted to the nearest integer.

TABLE II

Physicochemical properties of ABP

Molecular weight	
Dimer[a]	100,000
Monomers[b]	47,000
	41,000
Nonpolar residues[c]	51.3%
Carbohydrate (w/w)[d]	25.0%
Partial specific volume[c]	0.71 cm^3/g
Average hydrophobicity[c]	1013 cal/residue
Fractional charge[c]	0.334

[a] Data treated according Lamm (19).

$$\text{Molecular weight} = \frac{2RT}{(1-\bar{\nu}p)\omega^2} \cdot \frac{d\ln c}{dr^2}$$

where $\bar{\nu}$ is partial specific volume, p is density, ω is rotation rate, c is concentration, and r is the distance from center of rotation.

[b] Determined by SDS-polyacrylamide gel electrophoresis.

[c] Calculated from the amino acid composition.

[d] Analyzed by the method of Chambers and Clamp (12).

ABP antiserum prior to electrophoresis decreased ABP binding of [^{3}H]dihydrotestosterone. This method of detecting antibodies was described previously (17). We have not determined whether the decrease in binding resulted from antibody inactivation of the binding site on ABP or from failure of the antigen-antibody complex to enter the gel.

Antibody was evaluated further by immunocytochemical staining using the peroxidase technique (20). Specific staining for ABP was observed in cultured rat Sertoli cells, seminiferous tubules of rat testis, and in the epithelium of caput epididymis (Fig. 7). "Method specificity" (21) was tested by staining with increasing dilutions of anti-ABP serum. Optimal staining was obtained with dilutions from 1:1,000 to 1:10,000. No staining was detectable at dilutions of 1:100,000 or higher, indicating that staining depended on the primary antiserum and not on subsequent reagents or on endogenous peroxidase. As an indication of antibody specificity, staining was significantly reduced when anti-ABP serum was preabsorbed in the testis and totally eliminated in the epididymis with purified ABP or epididymis cytosol.

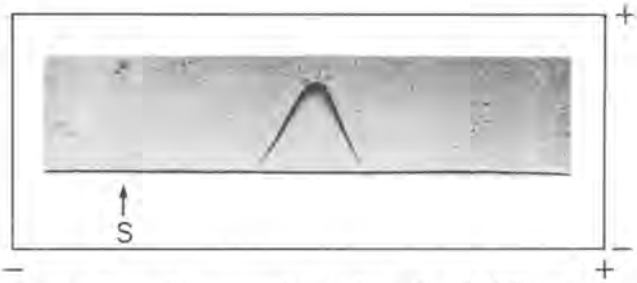

FIG. 5. **Crossed immunoelectrophoresis of ABP antiserum against purified ABP.** First dimension (*horizontal*), electrophoresis of 10 μg of purified ABP in a 2% agarose gel. *S* indicates the point of application. Second dimension (*vertical*), electrophoresis in a 1% agarose gel containing 0.5% anti-ABP rabbit serum.

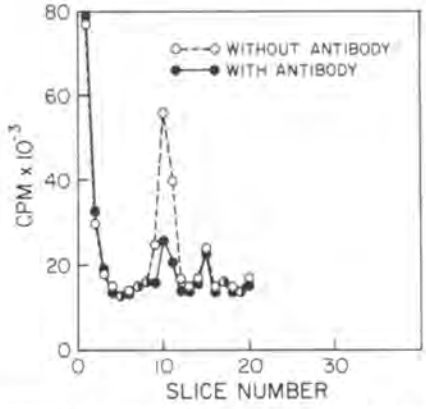

FIG. 6. **Antibody inhibition of ABP binding.** Rat epididymis cytosol (100 μl) labeled *in vitro* with 10 nM [^{3}H]dihydrotestosterone was incubated 18 h at 0 °C with 20 μl of anti-ABP rabbit serum diluted 1:100 and with normal rabbit serum as a control. Incubation mixtures were analyzed by polyacrylamide gel electrophoresis as described in the legend to Fig. 2. Presence of ABP antibody is indicated by the decrease in radioactivity bound to ABP.

Specific staining was localized to secretory granules of cultured Sertoli cells (Fig. 7*A*). In seminiferous tubules, immunoreactive ABP staining was observed in clusters of small granules at the basal and adluminal regions of Sertoli cell cytoplasm (Fig. 7*B*). Different segments of the same tubule varied greatly in their content of immunoreactive ABP. In epididymis (Fig. 7*C*), ABP was localized largely in the caput, where it was found to coat the microvilli and to be concentrated within the Golgi region of principal cells. These cells are known to contain numerous coated vesicles, secondary lysosomes, and multivesicular bodies which constitute morphological evidence for the function of absorption (23).

DISCUSSION

Purification of ABP to homogeneity from rat epididymis cytosol is made possible by affinity chromatography coupled with conventional methods of protein separation. Similar procedures have been used to purify steroid-binding proteins from plasma (24, 25). In preparing the affinity column, the 17-β-hemisuccinate ester of dihydrotestosterone is readily synthesized. We found the mixed anhydride method of coupling the steroid ester to 1,6-diaminohexane Sepharose more efficient and reproducible than the conventional carbodiimide method. Following the affinity chromatography step, albumin is the major contaminant and can be separated from ABP by gel filtration chromatography. ABP appeared to be homogeneous by electrophoresis in polyacrylamide gels of different pore size and by analytical ultracentrifugation. The molecular

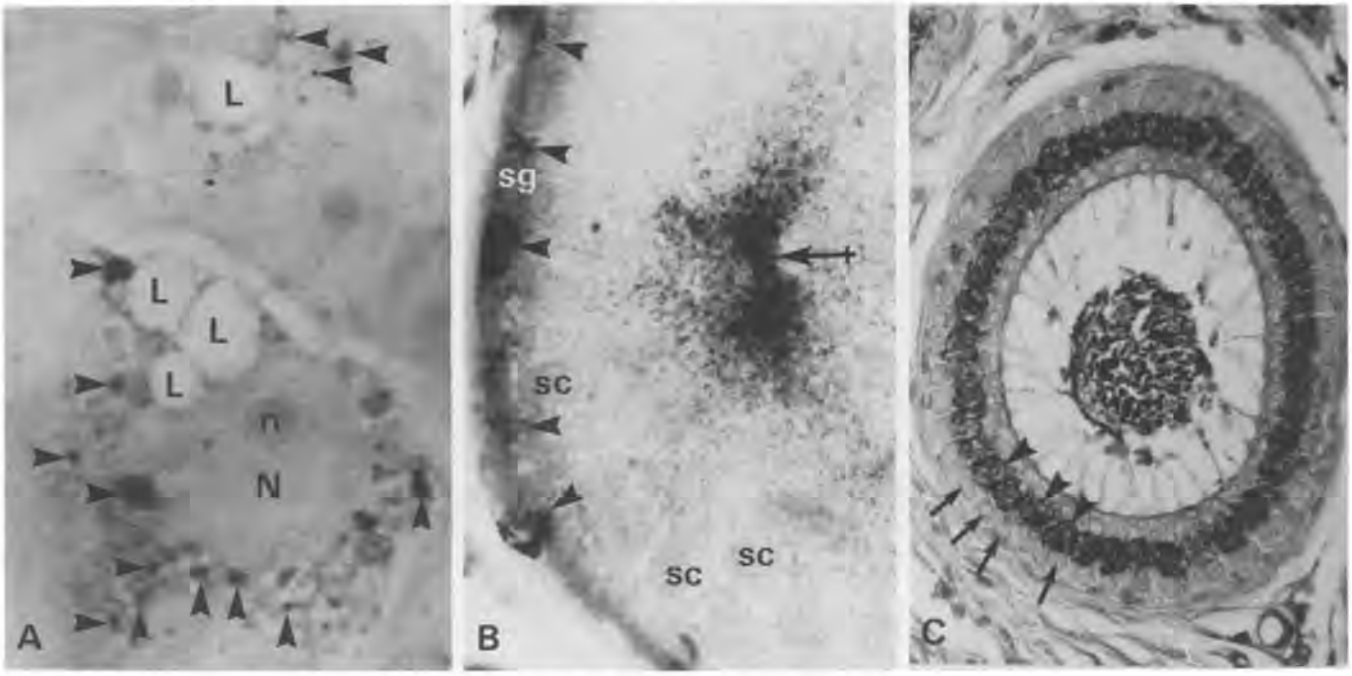

FIG. 7. **Immunocytochemical localization of rat ABP.** *A*, Sertoli cells cultured from 20-day-old rat testis (22) showing characteristic lipid droplets (*L*) and dark ABP immunoreactive granules of variable size, scattered throughout the cytoplasm. Some of the granules are indicated by the *arrowheads*. *N*, nucleus; *n*, nucleolus (× 3000). *B*, immunoreactive ABP in segment of seminiferous tubule from testis of a 28-day-old rat hypophysectomized 24 h and treated 48 h with follicle-stimulating hormone and testosterone. Peritubular space is to the *left*. Clusters of small, dark-staining granules are located in the basal (*arrowheads*) and adluminal (*arrow*) regions of the tubule, in presumptive Sertoli cell cytoplasm. Note lack of immunostaining in spermatogonia (*sg*) and spermatocytes (*sc*) (× 850). *C*, Cross-section of the epididymal duct from the region of the caput, showing immunoreactive ABP (*dark stain*) in the lumen and all epithelial cells lining the duct. In all epithelial cells, ABP staining is confined to the supranuclear (Golgi) region (*arrowheads*) of the cytoplasm. *Complete arrows* point to nuclei in the basal region of epithelial cells. Note lack of staining in the basal region of epithelial cells and in the surrounding smooth muscle and connective tissue (× 138).

weight of purified ABP as determined by sedimentation equilibrium is in reasonable agreement with previous estimates (90,000) on [^{3}H]dihydrotestosterone-labeled ABP in crude preparations, either by Ferguson plots of electrophoretic mobility in gels of different pore size (26) or by measurements of Stokes radius and sedimentation coefficient (27). Musto *et al.* (28) have recently reported a molecular weight of 85,000 for purified rat ABP as determined by sedimentation equilibrium, but obtained molecular weights of 111,000 to 154,000 from Ferguson plots of electrophoretic mobility.

Electrophoresis on SDS-polyacrylamide gels yielded two bands corresponding to molecular weights of 41,000 and 47,000 in a ratio of approximately 1:3. These two dissimilar subunits are identical in size and mass ratio to those reported by Musto *et al.* (28). ABP isolated from the rat testis has recently been shown to consist of the same subunits which, by peptide mapping, appear to be identical with the subunits of epididymal ABP (29). Moreover, it has been shown by photoaffinity labeling that ABP isolated from the medium of cultured Sertoli cells is composed of the same subunits (30). ABP purified from rabbit epididymis is reported to have a similar subunit structure (31). These findings suggest that the different subunits may result from posttranslational processing of ABP in Sertoli cells. Differences in the carbohydrate content of ABP subunits might account for their unequal size; however, this remains to be demonstrated. Our finding that ABP is a glycoprotein is in contrast to a report in which sugars could not be demonstrated by periodic acid-Schiff staining or amino acid analysis of purified ABP (28). Others, however, have recently reported that purified ABP does stain with periodic acid-Schiff stain and binds to concanavalin A (32). This property of rat ABP is shared by testosterone-binding globulin (24), a serum protein not present in the rat, but found in numerous other mammalian species (33). Indeed, it appears likely that ABP and testosterone-binding globulin are very similar proteins when they coexist in the same species (34).

ABP is secreted by Sertoli cells into the seminiferous tubular fluid and is carried through the efferent ducts into the caput epididymis where most of its binding activity is lost (1). Immunocytochemical studies indicate that ABP is largely absorbed by epithelial cells of the caput epididymis, where it has been shown previously that most of its binding activity is destroyed (1). A smaller portion of secreted ABP enters the blood stream (35); however, its route of entry has not yet been established. The striking accumulation of ABP in the basal portions of Sertoli cells suggests that its entry into blood may be by way of the interstitial fluid of the testis. Immunoperoxidase staining of cultured Sertoli cells indicates that ABP is packaged in secretory granules of variable diameter. Secretion of ABP containing granules occurs in response to follicle-stimulating hormone and is associated with striking changes in shape from flat to stellate or elongate morphology (22, 36, 37). *In vivo* ABP production is regulated by follicle-stimulating and androgenic hormones (7, 10); however, a variable content of immunoreactive ABP has been found in different segments along the same seminiferous tubule (38). Recent studies on isolated segments of seminiferous tubules have shown that Sertoli cell secretion of ABP varies in association with different stages of the spermatogenic cycle, being highest during the late stages of spermatid maturation (38). These observations suggest that hormonal stimulation of spermatogenesis is modulated by variation in functional activities of Sertoli cells (39). Control mechanisms intrinsic to the seminiferous epithelium may influence Sertoli cell responses to

follicle-stimulating hormone and androgens. The biological function of ABP remains to be determined; however, it could have local effects in the germinal epithelium associated with androgen action on spermatogenesis and more distal effects on epithelial cells in the caput epididymis. It has been suggested that ABP taken up by epithelial cells of the caput epididymis might serve to transport androgen from the testicular fluid to the cytoplasmic androgen receptor protein (1, 40).

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Additional references are found on p. 5175.

Supplement to

Androgen-binding Protein

By Mark Feldman, Oscar A. Lea, Peter Petrusz, Laura L. Tres, Abraham L. Kierszenbaum, and Frank S. French

This section describes the purification of ABP from rat epididymis.

Materials [1,2,4,5,6,7-^{3}H] dihydrotestosterone [^{3}H] DHT (123 Ci/mmole) was purchased from New England Nuclear, and checked for purity by thin layer chromatography. All chromatographic matrices (Sephadex G-25, Sephadex G-150 fine, DEAE-Sepharose, and AH-Sepharose 4B) were purchased from Pharmacia, with the exception of Bio-Gel HTP (hydroxylapatite) obtained from Bio-Rad Labs. 5 α-dihydrotestosterone (DHT) and succinic anhydride were from Sigma Chemical Co. as were bovine serum albumin, ovalbumin, and chymotrypsinogen A which were used as protein markers. Tri-n-butylamine and isobutylchlorocarbonate were purchased from Aldrich Chemical Co. Toluene, ether, dioxane (spectral grade), pyridine, and N, N^1-dimethylformamide (99 Mol% pure) were from Fisher.

Synthesis of [^{3}H] DHT-17β-hemisuccinate DHT (1450 mg), [^{3}H] DHT (25 μCi; 120 Ci/mmole) and 6 mmole succinic anhydride were dissolved in 10 ml toluene and refluxed overnight. The solution was partitioned between 250 ml each of ether and 0.01 N HCl (pH ≈2). The water phase was removed, and the ether re-equilibrated with an equal volume of 0.01 N HCl. The ether phase containing DHT-hemisuccinate was equilibrated with 500 ml of 0.8 M $NaHCO_3$. DHT-hemisuccinate is extracted into the $NaHCO_3$ solution, whereas free DHT remains in the ether phase. The aqueous phase was collected, and the ether phase equilibrated with $NaHCO_3$ in equal volume. The $NaHCO_3$ extracts were pooled and acidified with concentrated HCl to a pH of ≈2. This suspension was equilibrated with 250 ml of ether; the ether extract was collected and the ether evaporated. The residue was checked for purity against a DHT-hemisuccinate standard by thin layer chromatography on silica-gel F-254 in benzene; ethyl acetate (1:1) containing 1% acetic acid. Product yield was about 76%.

Coupling of [^{3}H] DHT-hemisuccinate to 1,6-diaminohexane-Sepharose (AH-Sepharose 4B) The mixed anhydride procedure (1,2) was adapted (Fig. 1). [^{3}H] DHT-hemisuccinate (430 mg, 16,000 cpm/mg) was dissolved in dioxane, 10 ml, and tri-n-butylamine 0.24 ml was added. The solution was cooled to 11°C, and 0.130 ml isobutylchlorocarbonate was added. The reaction was allowed to proceed for at least 20 minutes and mixed with 15 g AH-Sepharose in a volume of 100 ml of 1:1 dioxane/water plus 2 ml 1N NaOH (resulting pH 9.5). After 15 min, the pH fell and an additional 0.7 ml of 1N NaOH was added to bring the pH back to 8.5. Small amounts of 1N NaOH were added (0.2 ml, after a 30 min interval, and 0.05 ml after another 45 min) to maintain an alkaline pH. The reaction was allowed to proceed overnight and the AH-Sepharose was washed with dioxane on a sintered glass filter, until no radioactivity was detected in the eluate (at least 1 liter of dioxane). This was followed by washing with 80% methanol (1 liter) and finally distilled water (500 ml). The extent of steroid coupling was estimated by hydrolyzing 0.4 ml aliquots of the washed gel with 0.4 ml of 1 N NaOH at 37°C overnight followed by addition of 0.4 ml of 1N HCl. The gel was allowed to settle, and aliquots of supernatant counted in a liquid scintillation spectrophotometer. Knowing the specific activity of the steroid, moles of steroid/ml AH-Sepharose is easily calculated. Theoretical capacity for AH-Sepharose is 7-10 μmoles/ml.

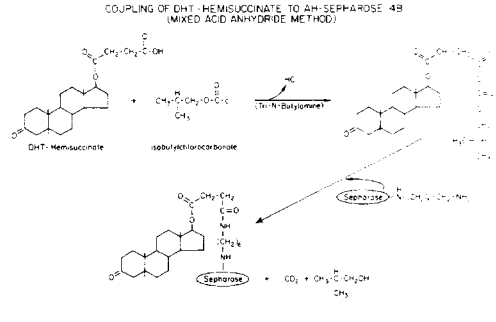

Figure 1

Figure 1 Mixed anhydride coupling of dihydrotestosterone-17β (DHT) hemisuccinate to 1,6 diaminohexane-Sepharose 4B.

Preparation of Cytosol Epididymis obtained from intact Sprague-Dawley rats was stored at -70°C. Caputs were separated from caudas and homogenized in 3 volumes 50 mM Tris, 10% glycerol pH 7.5 at 22°C (TG buffer) using an Omnimixer, 2 bursts of 40 sec each with 1 min periods of cooling between bursts. A batch of 350-400 g of caput was used in each purification and homogenized in 50g aliquots (150 ml TG buffer/aliquot). The homogenate was centrifuged in a type 35 rotor at 100,000 g for 70 min at 2°C. Supernatant was incubated with [^{3}H] DHT 0.2 nM for 1h. Protein concentration was measured by the method of Lowry et al (3). ABP binding activity was determined by polyacrylamide gel electrophoresis on 6.5% gels under steady state binding conditions as described by Ritzen et al (4). Cytosol preparations contained 10-14 g total protein and approximately 2 pmoles ABP/mg protein giving a total of 2-3 mg ABP as estimated from binding activity (4).

Purification of ABP Purification steps were carried out at 0-4°C. UV absorbance from column effluent was monitored at 280 nm using an ISCO UA-5 absorbance monitor equipped with a 5 mm flow cell.

Ion exchange chromatography was carried out on a DEAE Sepharose column (68 x 5 cm) equilibrated with TG buffer. [^{3}H] DHT labeled cytosol (1050 ml) was applied at a flow rate of 100 ml/h. Following application of the sample, the column was washed with TG buffer until the optical density of the eluate reached a constant baseline level. Absorbed proteins were eluted on a linear 0-0.6 M KCl gradient in 4000 ml TG buffer. Chloride ion was measured with a chloride ion electrode (Orion model 94-17). Fractions of 20 ml each were collected and aliquots removed to measure protein bound [^{3}H] DHT by liquid scintillation counting. A single peak of bound radioactivity eluted in 0.2-0.28 M KCl in TG buffer (Fig. 2). In this and subsequent purification steps the presence of ABP in radioactive peak fractions was confirmed by polyacrylamide gel electrophoresis (4).

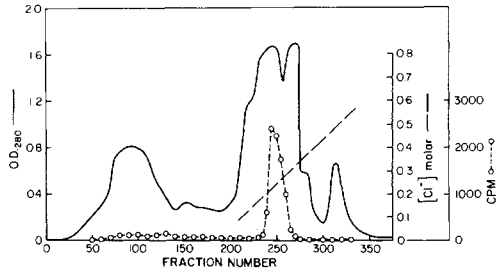

Figure 2 Ion exchange chromatography of rat epididymis cytosol on DEAE Sepharose after incubation with 0.2 nM [^{3}H] dihydrotestosterone. Labeled cytosol (1050 ml) was applied at a flow rate of 100 ml/h to a 68 x 5 cm column. The column was washed with 50 mM Tris, 10% glycerol pH 7.5 at 22°C (TG buffer) and adsorbed proteins eluted on a linear 0-0.6 M gradient in 4000 ml TG buffer. Protein was monitored by absorbance at 280 nm. Fractions of 20 ml were collected and aliquots removed for measurement of chloride ion and radioactivity.

Hydroxylapatite chromatography was performed in a column (15 x 7 cm) of Bio-Gel HTP (Bio-Rad laboratories) and equilibrated with TG buffer. Fractions from DEAE-Sepharose containing ABP (780 ml) were pooled and applied by gravitational flow at a column height of 15-30 cm. The top of the column was stirred gently 2-3 times during sample application to increase the flow rate. Following application of the sample the column was washed with TG buffer until the optical density reached a constant baseline. Adsorbed proteins were eluted with 0.2M KH_2PO_4/Na_2HPO_4 pH 7.0. Fractions of 20 ml were collected and [^{3}H] DHT-ABP detected by liquid scintillation counting. ABP eluted in the fall-through fractions (Fig. 3). However, elution of ABP varied somewhat with different lots of hydroxylapatite. ABP sometimes adsorbed to the column along with bulk proteins but could be eluted in low concentrations of phosphate buffer (5-10 mM) ahead of the major protein peak. This elution pattern was more often obtained when the sample was dialysed to remove salt prior to the hydroxylapatite step.

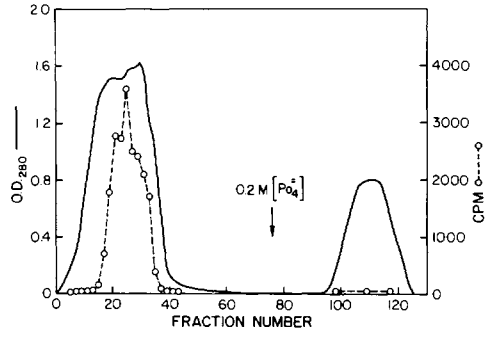

Figure 3 Elution profile on hydroxylapatite of protein and [^{3}H] dihydrotestosterone bound ABP. Fractions containing ABP from DEAE-Sepharose were pooled (790 ml) and applied to a 15 x 7 cm column of Bio-Gel HTP. Following sample application, the column was washed with TG buffer. Adsorbed proteins were eluted in 0.2 M KH_2PO_4/Na_2HPO_4 pH 7.0. Fractions of 20 ml were collected and aliquots removed for measurement of radioactivity.

Affinity chromatography was performed on an AH-Sepharose 4B column containing approximately 4 μmoles/ml of covalently linked [^{3}H] DHT-17β-hemisuccinate. The column was equilibrated in TG buffer containing 1M KCl. Pooled ABP fractions (750 ml) from the hydroxylapatite column were made to contain 1M KCl and applied at a flow rate of 75 ml/h following which the column was washed with TG buffer containing 1M KCl and 10% dimethylformamide (TGKD) (5) until a constant zero baseline level of absorbance was reached with UV monitor setting of 0.05 absorbance unit full scale (≈ 20 column volumes of buffer). ABP was eluted in 2-4 volumes of TGKD buffer containing 100 μM DHT (Fig. 4). During chromatography of the sample, there was no loss of covalently linked [^{3}H] DHT-17β-hemisuccinate suggesting that any esterase activity capable of cleaving the hemisuccinate linkage (5) was removed by the preceding purification steps.

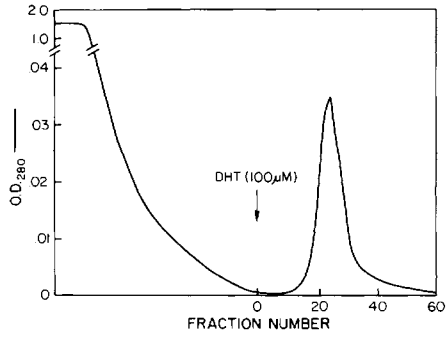

Figure 4 Affinity chromatography of ABP on a 1.6 x 25 cm column of [^{3}H] dihydrotestosterone-17β-succinyl-1,6-diaminohexane-Sepharose. Fractions from hydroxylapatite containing ABP were pooled (750 ml) and applied at a flow rate of 75 ml/h following which the column was washed with TG buffer containing 1M KCl and 10% dimethylformamide (TGKD). ABP was eluted in TGKD buffer containing 100 μM dihydrotestosterone and fractions of 5 ml were collected.

Protein eluting from the affinity column in TGKD buffer containing DHT was desalted on a Sephadex G-25 column (35 x 2.6 cm) equilibrated with TG buffer. Fall-through protein was concentrated on a small DEAE-Sepharose column (3 x 0.9 cm) equilibrated with TG buffer. Adsorbed protein was step eluted with a small volumne (≈4 ml) TG buffer containing 1M KCl.

Gel filtration chromatography was performed on Sephadex G-150. The concentrated protein from DEAE-Sepharose was incubated with 10 nM[^{3}H] DHT for 1 h at 0°C and chromatographed on a Sephadex G-150 column (95 x 1.6 cm) equilibrated with TG buffer. Fractions of 4.5 ml were monitored for protein and radioactivity (Fig. 5). ABP eluted in a symmetrical peak corresponding to a Stokes radius of 48 Å. The void peak contained aggregated ABP as judged by SDS-polyacrylamide gel electrophoresis. It was found that the amount of aggregated ABP was increased by lyophilization of the sample prior to gel filtration chromatography. The small peak eluting behind ABP had an electrophoretic mobility identical to albumin on SDS-polyacrylamide gels.

In a purification carried out subsequent to obtaining ABP antiserum, immunoreactive ABP was measured by radioimmunoassay (6). Recovery of immunoreactive ABP is shown in Table 1. The weight of final product is likely underestimated by the Lowry assay due to the low tyrosine content of ABP.

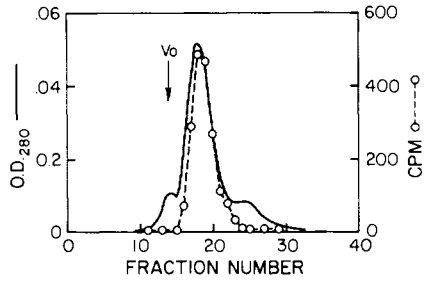

Figure 5 Gel filtration of partially purified ABP on Sephadex G-150. Peak fractions from the affinity column were pooled, desalted on Sephadex G-25 and concentrated on a small DEAE Sephadex column. The sample (4 ml) was labeled with 10 nM[^{3}H] dihydrotestosterone for 1h at 0°C and applied to a Sephadex G-150 column (95 x 1.6 cm) equilibrated with TG buffer. Fractions of 4.5 ml were collected and aliquots removed for measurement of radioactivity.

Table I

ABP Purification from Rat Epididymis

Step	Volume	(a) A Total Protein	(b) B ABP	Specific Activity B/A	Yield
	(ml)	(mg)	(mg)		
Cytosol	1200	14,400	5.5	0.0004	100
DEAE Sepharose	780	6,240	3.9	0.0006	71
Hydroxylapatite	750	2,400	3.2	0.0013	59
Affinity Chromatography	120	0.71	0.65	0.9200	12
G-150 Sephadex	27	0.41	0.49	1.2000	9

a.) Measured by the method of Lowry et. al. (3) using bovine serum albumin as the reference standard.

b.) Measured by radioimmunoassay (6).

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