

Purification of androgen-binding protein from rat testis using high-performance liquid chromatography and physicochemical properties of the iodinated molecule

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The androgen-binding protein (ABP) has been purified 87 500-fold from rat testis using 4 steps of HPLC, with a yield of 14%. The molecule was 99% pure with a specific activity estimated to 16 600 pmol/mg protein. The iodinated molecule was eluted in 2 peaks in Sephacryl S300 gel filtration with a molecular mass estimated to be $92\,600 \pm 3300$ and $50\,300 \pm 4000$ Da. The column isoelectrofocusing of ^{125}I -ABP demonstrated 3 isoproteins isoelectric at pH 4.7, 4.9 and 5.3 and the sedimentation coefficient was estimated to be 4.7 S in sucrose gradient ultracentrifugation. The ^{125}I -ABP had similar physicochemical properties to the non-labelled ABP of epididymis.

Androgen-binding protein (Testis) HPLC

1. INTRODUCTION

Two functional compartments can be described in the testis: the Leydig cell compartment which produces androgens and the seminiferous tubular compartment in which is secreted a fluid containing the spermatozoa. In the latter exocrine compartment the Sertoli cells have been found to contain several proteins, the synthesis of which is regulated by follicle-stimulating hormone and testosterone [1]. One of these proteins is ABP. The function and fate of ABP in seminiferous tubules and in the epididymis remain under discussion [2]. ABP has been identified from epididymis of

various mammals such as the rat, rabbit and sheep [2-7]. The first attempt to purify ABP has been carried out with rabbit epididymis using conventional chromatographic steps [8]. More recently, ABP has been purified from epididymis of various species using affinity chromatography as the first chromatographic step of the purification [9,11]. Until now, ABP has not been 100% purified from the testis. Recently, Larrea et al. [12] obtained a 47% purified preparation from rat testis, using affinity chromatography and preparative electrophoresis. To study the fate of ABP in the epididymis by autohistoradiography, we have purified ABP from rat testis and studied the physicochemical properties of the iodinated molecule. Since ABP is synthesized in the testis and hypothetically taken up by the caput of the epididymis, the study of the fate of this molecule has to be carried out using ABP purified from the testis rather than from epididymis.

Abbreviations: ABP, androgen-binding protein; HPLC, high-performance liquid chromatography; HP-SEC, high-performance size-exclusion chromatography; HPIEC, high-performance ion-exchange chromatography; DHT, 5α -dihydrotestosterone

2. MATERIALS AND METHODS

2.1. Chemicals

5 α -Dihydro[1,2,4,5,6,7-³H]testosterone (³H]-DHT) (180 Ci/mmol) and [¹²⁵I]iodine (100 Ci/mol) were obtained from the Radiochemical Centre (Amersham, England); guanidine hydrochloride, glycerol, acetonitrile and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt). Scintran^R (for scintillation counting) was obtained from BDH (Poole, England). Iodo-BeatsTM were purchased from Pierce (Rockford, USA). All other reagents were obtained as described in [13].

2.2. Preparation of testicular cytosol

Frozen rat testes (580 g) were homogenized in ice-cold 20 mM Tris-HCl buffer (pH 7.5) containing 10% glycerol (v/v) (TG buffer) with a tissue:buffer ratio (w/v) of 1:2 using a Polytron homogenizer. The homogenate was centrifuged at 17000 \times g for 2 h at 4°C, and the supernatant filtered through glass wool. ABP was precipitated from the testicular extract by addition of 0.36 g/ml of ammonium sulfate. The mixture was stirred for 12 h and centrifuged at 17000 \times g for 2 h at 4°C. The supernatant was saturated with 60% (NH₄)₂SO₄, stirred for 6 h and centrifuged again. The pellet was reconstituted in one-tenth its original cytosol volume, and consecutively dialysed against 20-fold the volume of TG buffer containing 5 M guanidine hydrochloride, 20-fold the volume of TG buffer containing 2.5 M guanidine hydrochloride, 10-fold the volume of TG buffer containing 1.5 M guanidine hydrochloride and renatured against 20-fold the volume of TG buffer. The sample was concentrated 10-fold in an Amicon ultrafiltration cell using a YM-10 membrane (Amicon, USA).

2.3. HPLC

HPLC was performed at room temperature using a two-pump gradient system (Water Associates, Milford, USA). The protein eluate was detected at 280 nm using a single-wavelength detector (Pharmacia, Uppsala) and collected in 1 ml fractions (Frac-100, Pharmacia). The sample injector was equipped with a 10 ml sample loop. The different parts of this system were interconnected by Teflon tubing. [³H]DHT was measured

by liquid scintillation spectrometry (Packard model 5780) using 5 ml Scintran^R with a counting efficiency of 30%.

2.4. HP-SEC

For determination of DHT-binding capacity in each step of the purification, bound DHT was separated from the free molecule using an Licrospher Diol 200 analytical column (0.7 \times 30 cm, Merck) eluted with 20 mM Tris-HCl buffer (pH 7.5) containing 10% glycerol (v/v) at a flow rate of 1 ml/min. 0.5 ml of the sample was injected per run. For the first chromatographic step of the purification of ABP, a preparative Diol column (2.5 \times 30 cm) was eluted at a flow rate of 10 ml/min and 10 ml of the sample was injected per run. No significant difference in elution profile was noted between the analytical and preparative columns.

2.5. HPIEC

The first HPIEC step was performed with a mono Q column (0.5 \times 5 cm, Pharmacia) eluted with TG buffer at a flow rate of 1 ml in a 0–1 M NaCl gradient. The first eluted radioactive fractions were pooled, dialyzed and concentrated to 3.1 ml using a YM-10 Diaflo membrane (Amicon). A part of the sample was incubated with 1 nmol [³H]DHT for 3 h at 4°C under rotative agitation and poured onto a mono S column (0.5 \times 5 cm, Pharmacia), eluted first with 5 ml of 10 mM citrate-HCl buffer (pH 8.1) and then with a 0–100% linear gradient of 20 mM ethanolamine (pH 9.2) containing 1 M NaCl in a total volume of 25 ml. The binding capacity of [³H]DHT was determined in each fraction using the analytical HP-SEC method as described above. The fractions containing ABP were pooled, dialyzed against distilled water containing 5% glycerol (v/v), concentrated and poured onto a C₈ reverse-phase affinity column (0.5 \times 5 cm, Pharmacia), eluted consecutively with 5 ml distilled water containing 0.05% TFA and with a 0–100% gradient of acetonitrile containing 0.05% TFA in a total volume of 30 ml at a flow rate of 0.5 ml/min. The collected fractions were analyzed for [³H]DHT-binding capacity as described above.

2.6. Iodination of ABP

Iodination of ABP was carried out according to

Markwell [14]. Two beads were washed and incubated successively in 0.2 ml of 50 mM Tris-HCl buffer (pH 7.8) with 0.1 mCi [¹²⁵I]iodine and 20 μg purified ABP. The iodinated molecule was poured, in the presence of dextran blue 2000, onto a Sephadex G-25 column (2.5 × 10 cm). The coupling efficiency was estimated from the proportion of bound [¹²⁵I]iodine.

2.7. Gel filtration

The [¹²⁵I]iodinated ABP was filtered through a Sephacryl S300 gel filtration column (1.0 × 50 cm) eluted with 20 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl and 3 mM NaN₃ at a flow rate of 30 ml/h. The column was calibrated and the molecular mass and Stokes radius estimated as described in [13].

2.8. Isoelectrofocusing

Liquid isoelectrofocusing was carried out in a 100 ml LKB column (type 8101, LKB, Bromma, Sweden) packed with carrier ampholytes of pH 2.5–4.0 and 3.5–5.0 in a final concentration of 1% and in a 0–50% sucrose gradient containing 3 M urea.

2.9. Sucrose density gradient ultracentrifugation

This was carried out with a 5–20% (w/v) linear sucrose gradient in a total volume of 5 ml per tube at 180000 × g and 4°C for 18 h. 0.1 ml labelled ABP sample was applied per tube. After centrifugation, 200 μl aliquots were collected and subjected to γ-ray counting and UV absorbance at

280 nm. The sedimentation coefficient was estimated using the reference molecules, bovine serum albumin, human serum albumin, IgG and aldolase.

3. RESULTS

ABP was purified 87500-fold from rat testes with a yield of about 14%. The specific activity of the testicular extract was 0.19 pmol DHT-binding sites/mg protein (table 1). Ammonium sulfate precipitation and dialysis enhanced the specific activity up to 4.04 pmol/mg proteins, demonstrating that a significant proportion of ABP was saturated by endogenous steroids in the initial testicular extract. The first step of HPLC eluted the [³H]DHT-ABP complex in two peaks (fig.1) which could correspond to the monomeric and dimeric forms of saturated ABP [3,15–18]. The purified molecule was iodinated with a yield of about 20% and a specific activity of 0.2 mCi/mg protein. The ¹²⁵I-ABP was eluted in 2 peaks in Sephacryl S300 gel filtration, with a molecular mass estimated to be 92600 ± 3300 Da and 50300 ± 4000 Da (*n* = 4), corresponding respectively to a dimeric and a monomeric form of ABP, as previously described [3,15–18]. The Stokes radii of the monomer and dimer were estimated to be 4.1 and to 2.95 nm respectively, using labelled human serum albumin as reference molecule. Column isoelectrofocusing demonstrated 3 isoproteins, isoelectric at pH 4.7, 4.9 and 5.3 (fig.2). The sedimentation coefficient of labelled ABP was estimated to be about 4.7 S

Table 1
Purification steps of ABP from rat testis

	Volume (ml)	DHT-binding capacity (pmol)	Protein content (mg)	Spec. act. (pmol/mg)	Purification factor	Yield (%)
Testicular extract	1468	2900	10.4	0.19	—	—
Ammonium sulfate precipitation and dialysis against guanidine hydrochloride	135	9446	17.3	4.04	21	100
Lichrospher Diol 200	7.9	6707	1.5	565	2970	71
Mono S	5.5	5097	0.41	2255	11870	54
Mono Q	3.1	3367	0.09	12014	63230	36
C ₈ reverse phase	2.0	1330	0.04	16625	87500	14

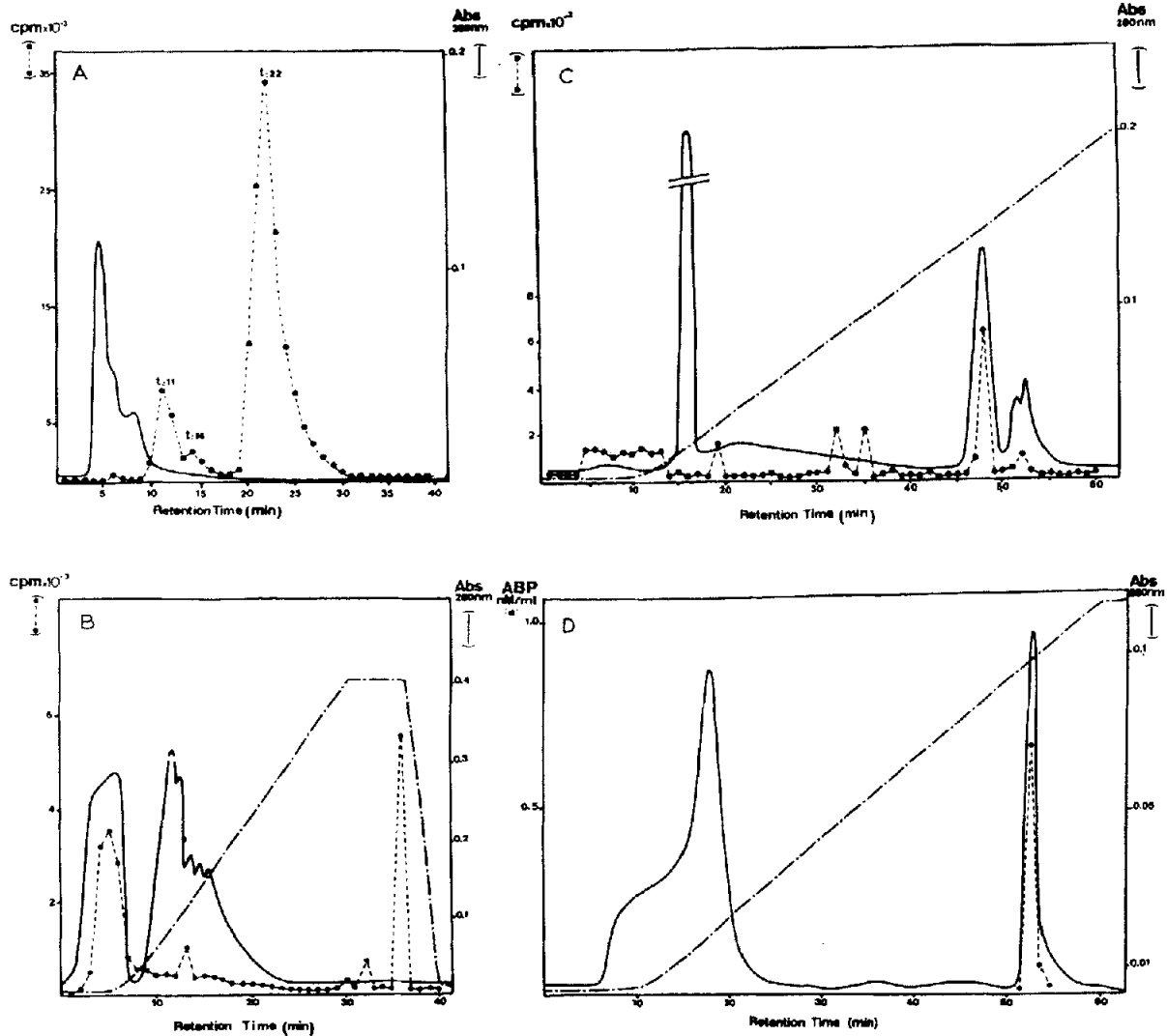


Fig.1. Elution profiles of each purification step of testicular ABP in HPLC. (A) HP-SEC of 50 μ l dialysed cytosol extract previously incubated with [3 H]DHT as described in section 2 and poured onto a Lichrospher Diol 200 column (0.7 \times 30 cm) eluted with TG buffer at a flow rate of 1 ml/min. Detection by absorbance at 280 nm and radioactivity of [3 H]DHT. ABP eluted with two retention times of 11 and 14 min, corresponding respectively to monomers and dimers. Free [3 H]DHT eluted with a retention time of 22 min. (B) HPIEC (column Mono Q) of 4 ml of the ABP fractions collected from the first step. The mobile phase was TG buffer with a linear gradient of 0–1 M NaCl in 25 ml. Flow rate and detection as above. Most of the ABP was eluted in the first fractions. (C) HPIEC (column Mono S) of 2 ml of the ABP fractions collected from the second step. The column was eluted successively with 0.01 M sodium citrate buffer (pH 1.8) and with a linear gradient of 0–100% of 0.02 M ethanolamine (pH 9.2) containing 1 M NaCl in 25 ml. Flow rate 0.5 ml/min, detection as above. ABP was mostly desaturated and eluted with a retention time of 48 min. (D) HPRPC (C_8 reverse-phase column) of the 2 ml fractions of ABP obtained from the third step. The column was eluted successively with water containing 0.1% (v/v) TFA and with a 0–100% gradient of acetonitrile containing 0.1% (v/v) TFA in a volume of 30 ml, at a flow rate of 0.5 ml. Detection by absorbance at 280 nm and DHT-binding capacity. ABP was eluted with a retention time of 53 min.

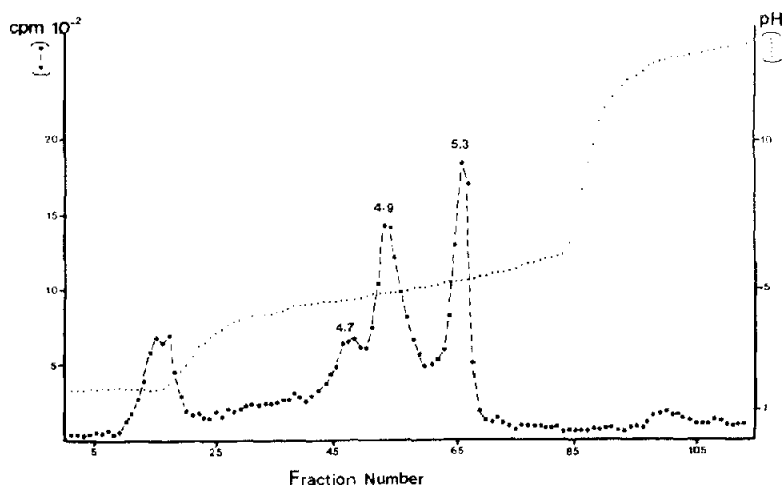


Fig.2. Isoelectrofocusing of ^{125}I -ABP demonstrated 3 isoproteins isoelectric at pH 4.7, 4.9 and 5.3 (●—●). The peak which focused in the beginning of the gradient was an aggregate of iodinated protein.

on sucrose gradient ultracentrifugation (fig.3). The physicochemical properties of the purified labelled ABP are summarized in table 2.

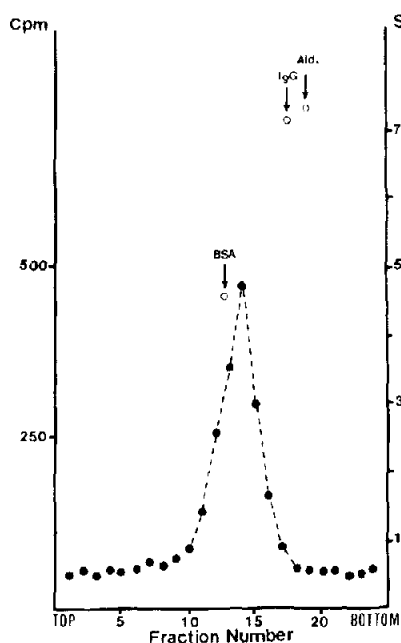


Fig.3. Sucrose density centrifugation of ^{125}I -ABP. The sucrose gradient was 5–20% (w/v). The position of standard proteins was detected by the absorbance at 280 nm. The position of ^{125}I -ABP corresponds to a sedimentation coefficient of 4.7 S.

Table 2

Physicochemical properties of ^{125}I -ABP

Molecular mass (Da)	$92600 \pm 3300/50300 \pm 4000$
Stokes radius (nm)	4.1/2.95
Sedimentation coefficient (S)	4.7
Isoelectric points	4.7, 4.9, 5.3
Specific activity (nmol/mg)	16.6

4. DISCUSSION

Treatment of the testicular extract by consecutive ammonium sulfate precipitation and dialysis against guanidine hydrochloride seemed to be much more efficient than that by consecutive ammonium sulfate precipitation and charcoal adsorption of testosterone [12] since the specific activity obtained by the first procedure was 4 pmol/mg proteins (table 1) instead of 0.4 pmol/mg proteins. In fact, the charcoal method is less efficient than dialysis against guanidine hydrochloride since (i) it can adsorb proteins as well as steroids and (ii) it is not a good method for removing the specifically bound steroids. Our results demonstrated therefore that a great proportion of ABP was saturated in the initial testicular extract (table 1).

HP-SEC was the first chromatographic step of our purification of ABP. This method was used not only for the purification, using a preparative Diol column, but also for the determination of the DHT-binding capacity of ABP, using an analytical column. This method was found to be much easier to perform than SDS-PAGE [19] and much more reliable than the charcoal method to determine the binding capacity of ABP [20]. In this way, the radiolabelled ABP was identified as dimers and monomers (fig.1). In the third and fourth HPLC steps, the use of a very acidic mobile phase induced significant removal of [^3H]DHT from ABP. The determination of DHT-binding capacity was therefore carried out using an HP-SEC method after neutralization of the collected fraction.

Assuming that a homogeneous preparation of ABP from rat epididymis had a specific activity of 16800 pmol/mg protein [10], our final ABP obtained from rat testes was found to be 99% pure with a specific activity of about 16600 pmol/mg proteins. In two recent works which used affinity chromatography as the first chromatographic steps, ABP has been partly purified from rat and human testes with a specific activity of 8000 and 12500 pmol/mg proteins, respectively [12,17]; the ABP from rat epididymis was 47% pure [12].

Our final preparation was considered 99% pure not only from its specific activity but also from its elution profile in reverse-phase HPLC (fig.1) and from the elution profile of the labelled molecule in gel filtration, column isoelectrofocusing and sucrose gradient ultracentrifugation (figs 2,3). The physicochemical properties of the purified iodinated ABP are very similar to those described by others [10,12], e.g. the formation of dimers and monomers and the microheterogeneity of the molecule with 3 isoproteins (table 2). This indicated that the iodination procedure did not induce any significant denaturation of the molecule.

In conclusion, we have purified 87500-fold the ABP from rat testes with a yield of 14% using 4 steps of HPLC. The molecule, which was 99% pure, was iodinated with a specific activity of 0.1 mCi/mg proteins and without any significant denaturation considering its molecular mass, microheterogeneity and sedimentation coefficient. This iodinated ABP will be used for its electron microscopic autohistoradiography localization in the epididymis after injection *in vivo*.

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