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PARTIAL PURIFICATION OF ANDROGEN BINDING PROTEIN FROM BULL EPIDIDYMIS

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Summary—An androgen binding protein (ABP), with an equilibrium dissociation constant of 4.2 nM and a molecular weight of about 100 kDa, has been purified from bull epididymal extracts using a four-step procedure. These preliminary results underline the main difficulties encountered in the purification of this protein present at a very low concentration (i.e. 50-fold less than in rat or rabbit epididymides). Ammonium sulfate precipitation is not a suitable step due to the formation, in presence of salt, of insoluble material leading to a loss of ABP. Lipids, particularly phospholipids, might be implicated in this phenomenon. Several steps, including anion exchange in batch followed by concentration, affinity chromatography and HPLC gel filtration allowed us to obtain a 7667-fold purified protein with a 9% yield.

INTRODUCTION

The androgen binding protein (ABP) is a Sertoli cell protein secreted in the lumen of the seminiferous tubules and transported to the epididymis. First described in the rat [1], this protein has since been found in many other species [2]. Its precise physiological role has not been elucidated, but this protein is probably involved in the maintenance of spermatogenesis by testosterone transfer [3]; indeed, high affinity binding sites for ABP on rat germ cell surface [4] and internalization of labelled ABP by rat epithelial epididymal cells [5] have been demonstrated.

Highly purified ABP has been first isolated from rat epididymis by several authors [6, 7] using bio-specific affinity chromatography with different ligands. Recently, Gueant *et al.*[8] have purified rat testicular ABP to the same specific activity (as compared to previous reports [6, 7]) with four HPLC steps and without affinity process. This protein was also purified from rabbit epididymis and its concentration is approximatively the same than in rat tissue extracts [9].

In order to (a) investigate an eventual physiological role of ABP in bull germ cell maturation and to (b) characterize it biochemically, we decided to purify this protein. Here we report the first purification of ABP from bull epididymis where it is present at a very low concentration compared to other models previously described [6, 7, 9].

MATERIALS AND METHODS

Chemicals and reagents

5α-Dihydro[1, 2, 4, 6, 7-^{3}H]testosterone (DHT), 6.66 TBq/mmol, was obtained from Amersham and radioinert DHT from Sigma chemical Co. (St Louis, Mo.). *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) was from Merck (Darmstadt, F.R.G.). DEAE–Trisacryl M and HMD–Ultrogel AcA 34 were purchased from IBF (France).

Cytosol preparation

Epididymides were collected from 2 yr-old bulls. They were carefully dissected from testicular albuginae, trimmed of fat and connective tissues, cut in very small pieces and homogenized with an Ultra-Turrax in two volumes of TEG buffer (10 mM Tris, 1.5 mM EDTA, 10% (v/v) glycerol, 0.02% NaN_3, pH = 7.5). This homogenate was centrifuged 10 min at 1200 *g* at 4°C and the supernatant was submitted to a 106,000 *g* ultracentrifugation for 1 h (4°C). The cytosolic fraction was filtered through glass wool before the first purification step.

Ammonium sulfate precipitation

Ammonium sulfate (390 g/l) was slowly added to the cytosol while the pH was maintained with the addition of NaOH. Following an overnight incubation, the precipitate was recovered by centrifugation at 17,500 *g* for 45 min and the pellet resuspended in TEG buffer. This solution was centrifuged again to remove the insoluble material.

Anion exchange step

DEAE–Trisacryl M with high capacity (100 mg BSA/ml of gel) was added to the cytosol in a ratio of 1 ml packed gel per 50 mg total protein, then layered on a Buchner and washed extensively with TEG buffer. The fraction containing ABP was obtained by eluting the anion matrix with 3 gel volumes of TEG buffer + 200 mM $(NH_4)_2SO_4$ and then concentrated using an YM30 Amicon membrane (Model 8400 cell).

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Affinity chromatography

The DHT–hexanoic acid (DHT–HA) ligand synthetized by Musto *et al.*[10] was coupled to the functional amino groups of HMD–Ultrogel AcA 34 (6-carbon spacer arm) using EEDQ as coupling agent. The reaction was carried out in 70% dioxane using 100 μg of DHT–HA and 5 mg of EEDQ/ml of gel, added twice, 5 h apart. The affinity matrix was washed extensively as previously described [9].

During the purification process, the sample was applied on the affinity gel at a low flow rate (12 ml/h) at 22°C and the column then washed with 10 bed volumes (Vt) of TDK buffer (20 mM Tris, 10% (v/v) dimethylformamide, 1 M KCl, pH = 7.5) at a 50 ml/h flow rate. The specific elution was run at 4°C with TDK buffer containing DHT (20 μg/ml), sequentially: two Vt elution—16 h incubation—two Vt elution—12 h incubation—and finally one Vt elution. The eluate was dialyzed against TEG buffer overnight and then concentrated on an Amicon YM 30 membrane.

Gel filtration

The molecular weight was determined using an Ultrogel AcA 34 column (1.5 × 77 cm) after loading a cytosolic sample and bABP was measured in each fraction (2.5 ml).

The gel filtration of the last purification step was realized on a TSK-SW-3000 (7.5 × 600 mm) column with a LKB HPLC system in a cold room at a 0.5 ml flow rate.

ABP assay

The androgen binding protein was quantified by measuring its binding capacity according to a method that we have recently described [11]. Briefly, samples are applied on a mini-column system, each column containing 500 μl packed DEAE–Biogel A. An extensive washing allows the removal of endogenous androgens. An incubation with tritiated 5α-DHT with (non specific) or without (total binding) a 500-fold excess of cold DHT was carried out inside the mini-columns. "Free" DHT was eluted in 1 min using a peristaltic pump with TEG buffer; "bound" DHT was collected after layering 3 × 200 μl of TEG buffer containing 200 mM NaCl and counted.

Lipid analysis

Lipids were extracted by the procedure of Folch *et al.*[12]. The phospholipid concentration was estimated by a modification of the Bartlett method [13]. The fatty acid methyl esters were obtained by transesterification of lipids with methanol-H_2SO_4 at 70°C [14]. After extraction with heptane, they were separated and identified by gas liquid chromatography using a capillary column Carbowax 20 M (50 m) with a temperature programmation (150–210°C at 1.5°C/min). Nonadecanoic acid was used as internal standard.

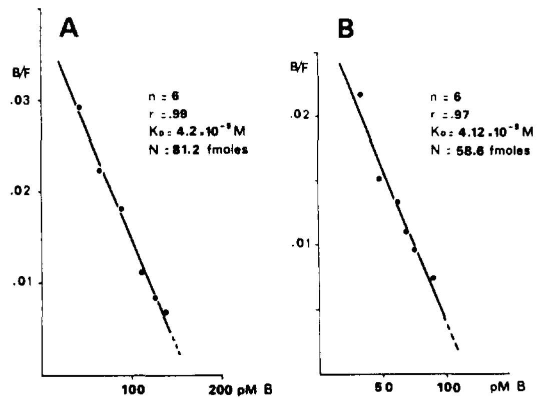

Fig. 1. Scatchard plot analysis of [^{3}H]DHT/ABP complex from rat (A) or bull (B) epididymis. Samples of 10 μl or 250 μl respectively for rat (A) and bull (B) epididymal cytosol were layered on the mini-columns (see Material and Methods) and then incubated for 2 h with increasing [^{3}H]DHT concentrations (1.25–20 nM) in presence or absence of a 500-fold excess of cold DHT.

Protein measurement

Protein concentration was measured by the dye binding method of Bradford[15] using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Partial characterization of cytosolic bABP

Scatchard analysis of bull cytosolic epididymal samples demonstrated the presence of a binding protein with a high affinity for labelled 5α-DHT. The equilibrium dissociation constant(K_d = 4.12 nM) gives the same value than that obtained, using the same technique, for epididymal rat ABP (K_d = 4.2 nM) (Fig. 1A and B). Repeated experiments

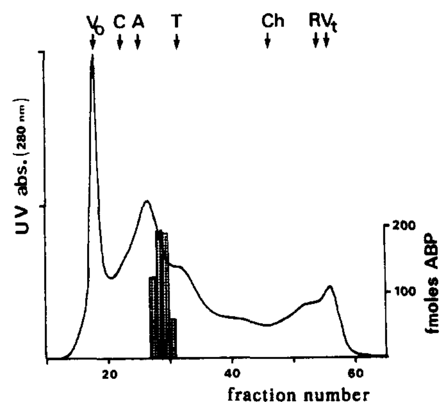

Fig. 2. Molecular weight determination of the cytosolic bull Androgen Binding Protein using an Ultrogel AcA 34 column (1.5 × 77 cm). The column was equilibrated and eluted with TEG bufier at a 26 ml/h flowrate in a cold room and ABP was measured in each 2.5 ml fraction. Standard proteins used for the column calibration were: catalase (C; 232,000), aldolase (A; 158,000), transferrin (T; 77,000), Chymotrypsinogen (Ch; 25,000) and ribonuclease (R; 13,700). Tritiated water and dextran blue were used to determine Vt and Vo respectively.

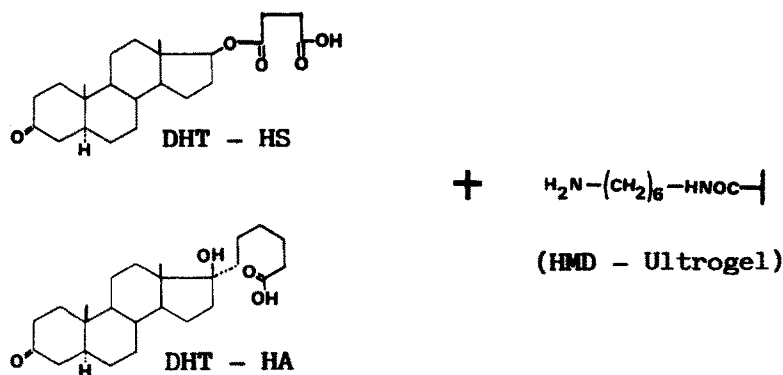

Fig. 3. The affinity matrix. Two types of ligand were successively used: (a) 5α-dihydrotestosterone, 17β-hemisuccinate (DHT–HS) and (b) 5α-dihydrotestosterone, 17α-hexanoic acid (DHT–HA). These affinity sorbents are coupled to diaminohexane-Ultrogel (HMD-Ultrogel) with a dihydroquinoline (EEDQ) as coupling agent.

yielded K_d values always ranging from 4 to 4.5 nM and from 3.9 to 4.7 nM, respectively, for rat and bull ABP. Molecular weight determination was performed on an AcA 34 gel filtration column and exhibited binding around 100 kDa (Fig. 2) in agreement with rat ABP data [16]. Furthermore, no loss of binding capacity was observed when the cytosolic sample was preincubated at 50°C for 30 min, which is a good criteria to distinguish ABP from a steroid receptor.

Specific activity

The first difficulty encountered in the purification of bull ABP is due to its very low concentration in this species. Our data for ABP specific activity in bull epididymis were around 40 fmols/mg of proteins and over 1600 fmols/mg of proteins in rat epididymis [11, 17]. Related values around 2000 fmols/mg of proteins were reported by authors in rat [6, 7] or rabbit [9] epididymis. So, there is about 50-fold less ABP in bull epididymal tissue when compared to other mammals. This interspecies variation appears more important when expressed as ABP content per gram of tissue, i.e. 368 and 40,600 fmols ABP/g of epididymis respectively in bull and rat, corresponding to a 110 ratio.

Affinity chromatography

Due to such a low concentration, a biospecific affinity step seemed essential. In preliminary experiments, an affinity matrix was prepared by synthetizing a 5α-dihydrotestosterone 17β-hemisuccinate (DHT–HS) ligand according to the Mickelson procedure [18], then coupled to diaminohexane-Ultrogel using EEDQ as described in Materials and Methods (Fig. 3); but the recovery was poor, not reproducible and decreased rapidly during repeated separations. A second affinity matrix has been prepared using a new ligand, 5α-dihydrotestosterone 17α-hexanoic acid (DHT–HA) [10] (Fig. 3), which gave better results (25–30% purification yield for this step) and could be used again.

Hemisuccinate steroid derivatives have been largely used for purification of vitamin D [19] or androgen [20] receptors, testosterone-estradiol binding globulin [18, 21] and also for rat ABP [4].

In fact, two hypotheses can be proposed to explain this second difficulty (a) steroids linked through hemisuccinate ester bond to the matrix can be cleaved by non specific esterases present in epididymal extracts [6, 10], and (b) linking DHT to an arm spacer at a 17α-position allows to keep "free" the 17β-hydroxy group which could play a role in a high affinity protein binding [22].

Ammonium salt precipitation

Ammonium sulfate was added to bull epididymal cytosol to a 60% saturation and the solubilized precipitate layered on the affinity column. Results presented in Table 1 underline the third difficulty: only about 40% of the ABP binding capacity was recovered after the ammonium sulfate step whereas in other models the yield was 77–95% [6, 9].

In order to localize the ABP precipitation peak, fractions of an epididymal cytosol were treated with increasing ammonium sulfate concentration from 30 to 80%. Surprisingly, except for the lowest concentrations, there was always proportionally less precipitated ABP than total proteins leading to a purification factor constantly < 1. In fact, the missing ABP was only partially recovered in the supernatant fraction (from 50 to 80%) and thus is mainly included

Table 1. Purification of bull epididymal ABP: fiirst procedure

Step	Specific activity[a]	Yield (%)	Purification factor
Cytosol	32.5	100	1
$(NH4)_2SO4$ ppt (60% sat)	26.4	40	0.8
Affinity Chromatography	46,264	10 (24.7)[b]	1400 (1750)[b]

[a]Specific activity is expressed as fmols of [^{3}H]DHT bound/mg protein; [b]results in parentheses are related to the previous step.

Table 2. Lipid distribution after salt precipitation

Sample	Proteins (%)	Phospholipids (μg/mg Protein)	(%)	Fatty acids (μg/mg Protein)	(%)
Cytosol	100	27.4	100	28.8	100
Supernatant	16	1.4	1	28.1	16
Precipitate (free of insoluble material)	74	6.9	19	9.5	24
Insoluble material[a]	10	221	80	173	60

[a]Values in this fraction were deducted from calculation.

Table 3. Purification of bull epididymal ABP: second procedure

Step	Protein (mg)	ABP (fmols)	Specific activity[a]	Yield (%)	Purification factor
Cytosol	10,622	403,636	38	100	1
DEAE–Trisacryl	3515	276,750	78.8	68.5	2.07
Affinity chromatography	0.694	77,490	111,697	19.2 (28)[b]	2940 (1420)[b]
GP–HPLC	0.125	36,420	291,360	9 (47)[b]	7667 (2.6)[b]

[a]Specific activity is expressed as fmols of [^{3}H]DHT bound/mg protein; [b]results in parentheses are related to the previous step.

in the insoluble material that was formed in presence of salt and was eliminated during the second centrifugation. This material is heterogeneous and partly represented by lipid components: diethylether extraction produces a gelatinous pack inside the organic phase. To get more precision on the nature of these components, total fatty acids (esterified and non esterified) and phospholipids were assayed in cytosol and in parts of salt fractionation, but also in rat epididymal cytosol. Results demonstrate (a) that the fatty acid concentration was 2-fold higher in bull than in rat cytosol (respectively 28.8 and 14.2 μg/mg proteins), (b) that the phospholipid concentration was 3-fold higher in bull than in rat cytosol (respectively 27.4 and 9.5 μg/mg proteins), (c) after salt fractionation, only 40% of fatty acid and 20% of phospholipids were present in the supernatant and in the solubilized pellet free of insoluble material (Table 2).

Probably ABP, known to exhibit non polar properties due to its amino acid composition [7], was partly carried by hydrophobic interactions with components (mainly phospholipids, quantitatively more important in bull than in rat cytosol and largely concentrated in the insoluble material fraction) which floculated in presence of salt. Such affinity between steroid binding proteins (plasma globulins or receptors) and lipids (fatty acids) have been demonstrated by Nunez *et al.*[23] and Vallette *et al.*[24].

Purification process

A second procedure, in which the ammonium sulfate precipitation has been replaced by an anion exchange step in batch, allowed a better ABP recovery (near 70%) and a 2-fold increase in specific activity (Table 3). After concentration and affinity chromatography, a 2940 purification factor was obtained versus 1400 in the original procedure and a 2-fold better ABP recovery. HPLC gel filtration yielded a 7667-fold purified protein by collecting only

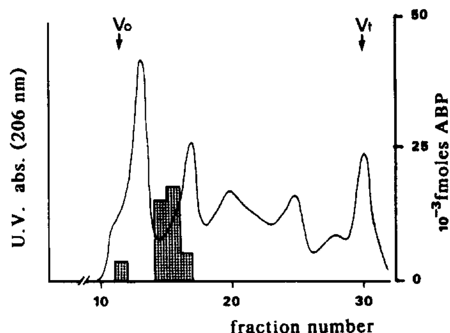

Fig. 4. Fourth purification step by HPLC-gel filtration. After salt and cold DHT removal by dialysis against TEG buffer and concentration on YM30 Amicon membrane, proteins collected from affinity chromatography were layered on a TSK-SW-3000 column (600 × 7.5 mm)—1000–300,000 MW range—and eluted at a 0.5 ml/min flow rate. 1 ml fractions were collected and ABP was measured in each of them (hatched columns). For the purification process, aggregative forms (in the void volume) and fraction 17 were eliminated.

fractions 15 and 16 [Fig. 4]. Compared to ABP purification in other species, our purification factor is 2.5-fold higher [7] or in the same order [6, 9] but the final specific activity was different related to the initial concentration in tissue extracts.

In conclusion, the present data described the main difficulties in purifying bull epididymal ABP (a) a very low concentration in tissue extracts, (b) the choice of ligand for more reliable affinity step, and (c) the presence of cytosolic components which makes ammonium sulfate precipitation not suitable. Nevertheless, bull androgen binding protein was nearly 7700-fold purified with a 9% yield.

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REFERENCES

1. Ritzen E. M., Nayfeh S. N., French F. S. and Dobbins M. C.: Demonstration of androgen-binding components in rat epididymis cytosol and comparison with binding components in prostate and other tissues. *Endocrinology* **89** (1971) 143–151.
2. Carreau S.: L'Androgen Binding Protein (ABP) chez le rat, le bélier, les taureau et l'homme: analyse comparée. In *Binding Proteins of Steroid Hormones* (Edited by M. G. Forest and M. Pugeat). INSERM/John Libbey Eurotext Ltd, Vol. 149 (1986) pp. 293–303.
3. Bardin C. W., Cheng C. Y., Musto N. A. and Gunsalus G. L.: The Sertoli cell. In *The Physiology of Reproduction* (Edited by E. Knobil and J. Neill). Vol. 21 (1988) pp. 933–964.
4. Dighe R. R., Meistrich M. and Steinberger A.: Sertoli-germ cell interaction: uptake of Sertoli cell secretory proteins by the germ cells during spermatogenesis. In *Development and Function of the Reproductive Organs* (Edited by A. Eshkol, B. Eckstein, N. Dekel, H. Peters and A. T. Tsafriri) SSR No. 11 (1986) pp. 161–170.
5. Gerard A., Khanfri J., Gueant J. L., Fremont S., Nicolas J. P., Grignon G. and Gerard H.: Electron microscope radioautographic evidence of *in vivo* androgen binding protein internalization in the rat epididymis principal cells. *Endocrinology* **122** (1988) 1297–1307.
6. Musto N. A., Gunsalus G. L. and Bardin C. W.: Purification and characterization of androgen binding protein from rat epididymis. *Biochemistry* **19** (1980) 2853–2860.
7. Feldman M., Lea O. A., Petrusz P., Tres L. L., Kierszenbaum A. L. and French F. S.: Androgen binding protein. Purification from rat epididymis, characterization immunocytochemical localization. *J. Biol. Chem.* **25** (1981) 5170–5175.
8. Gueant J. L., Khanfri J., Gerard H., Fremont S., Gerard A., Grignon G. and Nicolas J. P.: Purification of androgen binding protein from rat testis using hight performance liquid chromatography and physiochemical properties of the iodinated molecule. *FEBS Letts* **207** (1986) 280–286.
9. Cheng S. L. and Musto N. A.: Purification and characterization of androgen binding protein from rabbit epididymis. *Biochemistry* **21** (1982) 2400–2405.
10. Musto N. A., Gunsalus G. L., Miljkovie M. and Bardin C. W.: A novel affinity column for isolation of androgen binding protein from rat epididymis. *Endocr. Res. Commun.* **4** (1977) 147–157.
11. Barbey P., Fradin S., Carreau S. and Drosdowsky M. A.: A mini-column apparatus for Androgen binding protein measurement. *Analyt. Biochem.* **16** (1987) 167–173.
12. Folch J., Lees M. and Shone Stanley G. H.: A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226** (1957) 497–509.
13. Bartlett G.: Phosphorus assay in column chromatography. *J. Biol. Chem.* **234** (1958) 466–468.
14. Hagenfeldt L.: A gas chromatographic method for the determination of individual free fatty acids in plasma. *Clin. Chim. Acta* **13** (1966) 266–268.
15. Bradford M. M.: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72** (1976) 248–254.
16. Hansson V., Ritzen E. M., French F. S. and Nayfeh S. N.: Androgen transport and receptor mechanisms in testis and epididymis. In *Handbook of Physiology* (Edited by D. W. Hamilton and R. O. Greep). Am. Physiol. Soc., Washington, D.C., Vol. 5, Sect. 7 (1975) pp. 173–201.
17. Lecacheux P., Carreau S., Drosdowsky M. A., Nibart M., Abbel Malak G. and Thibier M.: Androgen binding protein, sperm production, semen output and LH and testosterone profiles in young post pubertal bulls. *Theriogenology* **15** (1981) 477–490.
18. Mickelson K. E. and Petra P. H.: Purification of the sex steroid binding protein from human serum. *Biochemistry* **14** (1975) 957–963.
19. Sharpe R., Hillyard C. J., Szelke M. and Macintyre I.: Purification of chick 25-hydroxycholecalciferol binding proteins by affinity chromatography. *FEBS Letts* **75** (1977) 265–271.
20. Chang C. H., Rowley D. R. and Tindall D. J.: Purification and characterization of the androgen receptor from rat ventral prostate. *Biochemistry* **22** (1983) 6170–6175.
21. Mickelson K. E. and Petra P. H.: Purification and characterization of the sex steroid binding protein of rabbit serum. *J. Biol. Chem.* **253** (1978) 5293–5298.
22. Petra P. H. and Lewis J.: Modification in the purification of the sex steroid binding protein of human serum by affinity chromatography. *Analyt Biochem.* **105** (1980) 165–169.
23. Nunez E. A.: Rôle des acides gras sur les intéractions entre protéines et stéroïdes. In *Binding Proteins of Steroid Hormones* (Edited by M. G. Forest and M. Pugeat). INSERM/John Libbey Eurotext Ltd, Vol. 149 (1986) pp. 415–426.
24. Vallette G., Christef N., Bogard C., Benassayag V. and Nunex E. A.: Dynamic pattern of estradiol binding to uterine receptors of the rat. Inhibition and stimulation by unsaturated fatty acids. *J. Biol. Chem.* **263** (1988) 3639–3645.