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CHARACTERISTICS OF THE CALF UTERINE ANDROGEN RECEPTOR

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Summary—The highest molecular weight form of the calf uterine androgen receptor separates as an 11S form in glycerol gradients. This "cytosolic" receptor, prepared in the presence of molybdate, polyethyleneimide and low ionic strength, dissociates into 9S and 7.2S forms with increasing KCl concentration. A 4.5S androgen binding component appears as the predominant form of the receptor in the absence of polyethyleneimide and this unit quantitatively converts to a stable 3.5S form in the absence of molybdate. Renaturation of partially purified protein, separated by SDS–PAGE electrophoresis, demonstrates the presence of an androgen binding component in the 110 kDa region of the gel. This renatured protein separates as a 4.5S component in glycerol gradients and has a Stokes radius of 6 nm.

Photoaffinity labelling of partially purified receptor preparations, followed by SDS–PAGE electrophoresis, reveals the presence of an androgen binding component having a molecular weight of 115 kDa. The binding characteristics and specificity of the receptor binding to R1881 have been studied and a DHT-affinity chromatography resin used to purify the receptor.

INTRODUCTION

The clonage and sequence analysis of the human androgen receptor [1, 2] has shown that it belongs to the steroid receptor subfamily that includes the mineralocorticoid, glucocorticoid and progesterone receptors [3,4]. These receptor genes have characteristic sequence homologies, a similar size and the receptors bind to the same hormone receptor elements in the promotor LTR of the retrovirus MMTV [5–8]. This work has helped to resolve some of the ambiguity concerning the size and structure of the androgen receptor, resulting from the various attempts to characterise the receptor by protein purification [9–12]. Its purification has proved difficult in that it is present at relatively low concentrations in target tissues and is relatively unstable. However, in order to understand the mechanism of action of androgen in normal tissues and neoplasms [13, 14] a more detailed knowledge about the structure and properties of the androgen receptor is required.

There have been many studies of the androgen receptor in various species and target tisues and more consistent results have now appeared [15–17] suggesting that the basic molecular form of the receptor has a molecular weight of about 115 kDa, a conclusion that supports the earlier work of Wilson and French [11]. A characterisation of the monomeric androgen receptor in several tissues has been described by Johnson *et al.* [16]. Higher molecular weight forms of the receptor have been observed by Colvard and Wilson [15] that they believe result from an association of the receptor (120 kDa) with a non-steroid binding protein component.

We now present the results of our own work on this receptor that shows some interrelationships between the high molecular weight forms and the monomeric form of the receptor.

METHODS

Cytosol preparation

Calf uteri obtained from immature animals, that had not been subject to treatment with steroid implants, were homogenised at 0°C, in a low ionic strength buffer containing 10 mM phosphate, 20 mM molybdate, 5 μM triamcinolone acetonide, 10% glycerol and 0.1 thioglycerol. The homogenate was centrifuged at 200,000 g for 1 h and the resulting cytosol (supernatant) fraction collected. The protein concentration of this cytosol was measured using Folin reagent.

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Binding studies

Cytosol preparations were preequilibrated with [^{3}H]R1881 (5 nM) in the presence of varying concentrations of R1881 (methyltrienolone), progesterone, estradiol and testosterone. The displacement of labelled [^{3}H]R1881 (86 Ci/mmol) from the receptor was determined using dextran-coated charcoal assay techniques.

A Scatchard plot for the binding of [^{3}H]R1881 was obtained after incubating 500 μl cytosol overnight with various concentrations of [^{3}H]R1881 (in the range 0.1–5 nM), 1 nM [^{3}H]R1881 plus 100 μM R1881, 5 nM [^{3}H]R1881 plus 500 μM R1881. The amount of bound radioactivity was then measured using dextran-coated charcoal or hydroxyapatite assay techniques and the results used to obtain Scatchard plots.

Polyethyleneimine precipitation

Samples of cytosol (50 ml) were equilibrated at 0°C with 1 nM [^{3}H]R1881 or [^{3}H]estradiol and polymin (polyethyleneimine) added, with rapid stirring, every few seconds sufficient to increase the concentration of cytosol polymin sequentially by 0.0025% up to a final concentration of 0.05%. After each addition a 100 μl aliquot was taken into an eppendorf tube and at the end of 5 min these tubes were rapidly centrifuged and the radioactivity remaining in each supernatant determined. The decrease in radioactivity remaining in solution was measured in order to determine the amount of receptor precipitated. The experiment was repeated allowing 30 or 120 min for receptor precipitation, using the same rapid mixing and sampling technique to vary the polymin concentration.

Partial purification

A large scale homogenisation routine (at 0–4°C) was developed in which deep frozen uteri (300 g) were broken into small pieces and homogenised in 1200 ml of uterine buffer, using a Waring Blender. The homogenate was then centrifuged at 10,000 ***g*** for 15 min to remove fibrous tissue and cell debris and then at 50,000 g for 90 min to obtain 1200 ml cytosol containing about 1 pmol/ml androgen receptor. Polymin (0.015%) was added to the cytosol followed by centrifugation at 10,000 ***g*** for 5 min, a procedure that precipitates the majority of the cytosol protein while leaving the majority of the androgen receptor in solution, providing a 10–20-fold purification of the androgen receptor. The androgen receptor was precipitated from this solution by adding more polymin (0.04%) followed by centrifugation at 10,000 ***g*** for 5 min. The androgen receptor was recovered from the pellet by shaking with uterine phosphate buffer for 5 min, a procedure in which most androgen receptor was recovered into the solution but most other protein remains in the pellet, a technique somewhat similar to that used for purification of recA protein [18]. Total protein precipitation could not be measured by standard assays, due to polymin interference, so was estimated simply by weighing the precipitated protein.

Glycerol gradients

Uterine buffer cytosol was incubated, under various ionic conditions, at 4°C overnight in the presence of [^{3}H]R1881 (5 nM) and 200 μl aliquots run on glycerol gradients (5–35%) using a ultracentrifuge at 200,000 ***g*** for 12 h. Glucose oxidase was added to each sample to act as a marker in determining the sedimentation coefficients.

High-pressure liquid chromatography

Samples of [^{3}H]R1881 (5 nM) labelled receptor (200 μl) were separated by high-pressure liquid chromatography (HPLC) using a Packard Column (W3000) and eluting with uterine cytosol buffer, with or without 0.4 M KCl. A calibration curve for a range of standard proteins was used to determine equivalent Stokes radii for the various binding components separated.

Protein renaturation

A polymin extract containing 100 pmol of receptor was precipitated with 10% TCA and subjected to SDS–PAGE electrophoresis after which the gel was sectioned and protein extracted and renatured, following procedures outlined by Sakai and Gorski [19], in the presence of [^{3}H]R1881. The bound radioactivity in each section was then determined using dextran-coated charcoal to remove free steroid. The renatured sample was characterised by HPLC and glycerol gradient separation. The experiment has been repeated several times and renaturation studied as a function of dilution and time.

Photoaffinity labelling

Polymin extract samples were preequilibrated with a saturating concentration of [^{3}H]R1881

(5 nM) and 1 ml aliquots were held at 0°C using a cryostat and illuminated for up to 5 min using a mercury high pressure lamp, surrounded by a water heat filter, placed 2 cm above the sample. The protein in the sample was then precipitated with 10% TCA and subjected to SDS–PAGE electrophoresis in columns (0.55 × 15 cm). The gel was sectioned and the radioactivity in each section determined. Corresponding experiments were carried out with the same receptor preparation preequilibrated with [^{3}H]R1881 (5 nM) in the presence of 1 μM testosterone.

Affinity chromatography

A large scale homogenisation routine (at 0–4°C) was carried out, as previously described, in order to obtain 1200 ml cytosol containing about 1 pmol/ml androgen receptor. Polymin (0.015%) was added and precipitated protein removed by centrifugation at 10,000 *g* for 5 min. The supernatant was then passed slowly (20 ml/h) through a DHT-affinity resin [20] column. Afterwards the resin was washed with buffer (5 × vol) with 0.2 M KCl (5 × vol) once more with buffer (5 × vol) with 2 M urea (5 × vol) and finally with buffer (5 × vol).

Receptor elution from the resin was achieved using an equal volume of buffer containing a high R1881 (100 μM) concentration with a tracer amount of [^{3}H]R1881, at room temperature, for 90 min with gentle agitation. The resin suspension was cooled to 0°C filtered through a Buchner funnel and the filtrate then passed slowly through a small heparin Sepharose column (0.5 ml). The heparin Sepharose was washed with 20 ml buffer and then twice with 1 ml aliquots of 0.3 M KCl. The receptor was recovered in 1 ml KCl (1 M) and SDS–PAGE electrophoresis of 20 μl aliquots carried out along with a set of protein standards run and silver stained at the same time. The radioactivity recovered was used to estimate the amount of receptor present in the eluate and the protein estimated by silver staining.

RESULTS

The antiglucocorticoid, triamcinolone acetonide (5 μM) effectively displaces all [^{3}H]R1881 bound to the uterine progesterone receptor without displacing [^{3}H]R1881 from the androgen receptor. Studies on the androgen receptor were subsequently always performed in the presence of 5 μM triamcinolone acetonide. The relative specificity of the binding was assessed in relation to the poor ability of estrogen and progesterone, but good ability of testosterone to displace [^{3}H]R1881 from the receptor (Fig. 1).

The binding characteristics of [^{3}H]R1881 in calf uterine cytosol studied under displaceable conditions using unlabelled R1881 demonstrate after Scatchard plot analysis the presence of a single set of binding sites and a dissociation constant of 0.35 nM with receptor concentration 100 fmol/mg protein. Similar binding characteristics were obtained if hydroxyapatite was used to measure receptor bound [^{3}H]R1881. The [^{3}H]R1881 binding protein separated as a single band on glycerol gradients (Fig. 2) with a peak corresponding to a sedimentation coefficient 4.5S and a shoulder corresponding to 3.5S. The sedimentation was affected by removing molybdate in that a single peak predominates, having a sedimentation coefficient

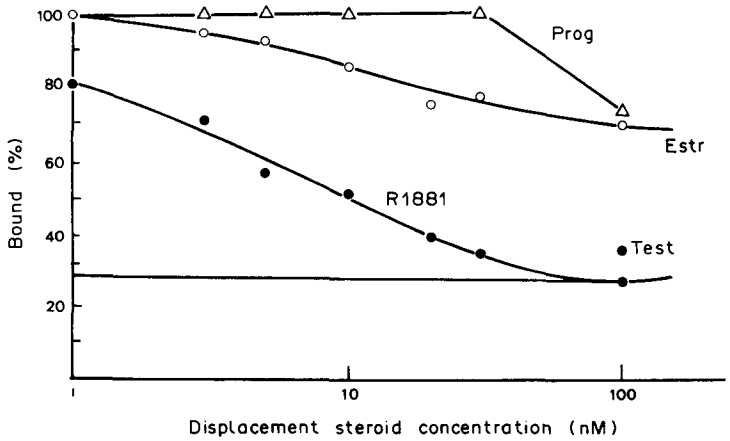

Fig. 1. Shows the relative efficiency of various steroids in displacing bound radioactivity from [^{3}H]R1881 (5 nM) equilibrated cytosol. Binding assays were carried out using standard dextran-treated charcoal assay routines.

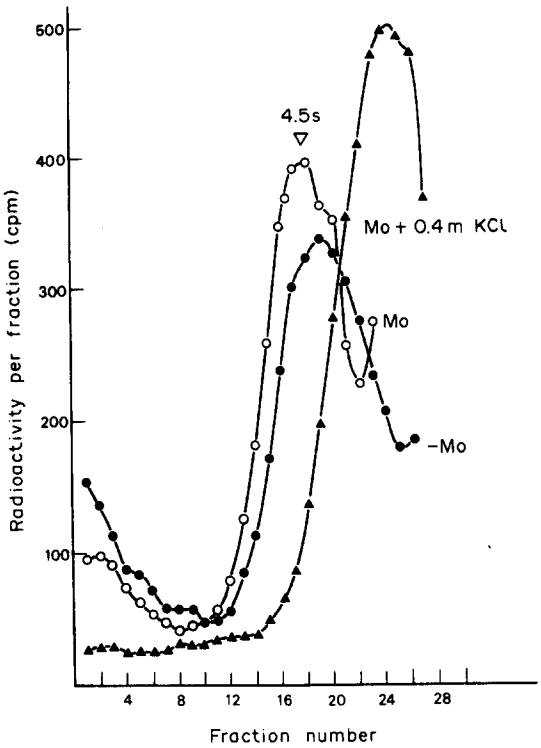

Fig. 2. Uterine buffer cytosol incubated overnight in the presence of [^{3}H]R1881 (5 nM). Aliquots (200 μl) were run on glycerol gradients (10–35%) under various ionic conditions, using an ultraspeed centrifuge at 200,000 g for 12 h. The sedimentation coefficients corresponding to the peaks of radioactivity were estimated relative to the peak of GO activity run in samples as a marker.

corresponding to 3.5S. In the presence of 0.4 M KCl the receptor breaks down but in low salt buffer the 4.5S receptor was found to be stable for up to 4 days in a cold room at 4°C.

After protein precipitation for 5 min using 0.015% polymin, some 80% of the androgen receptor remained in solution whereas the majority of the other protein was precipitated. When the concentration of polymin was increased to (0.04%) the androgen receptor was precipitated but could be recovered (60%) by five sequential washing of the pellet in 20 ml of buffer or a single wash in 100 ml buffer. When the resolubilised receptor was equilibrated with [^{3}H]R1881 and separated on glycerol gradients, in low ionic strength buffer, then a stable 11S form was consistently evident (Fig. 3). This dissociated to give a 7.2S form when separated on glycerol gradients in the presence of 0.4 M KCl. Similarly, the androgen receptor present in the supernatant obtained following polymin (0.015%) precipitation of cytosol separated with bound [^{3}H]R1881 as an 11S form on glycerol gradients. This 11S form dissociates in the presence of 0.15 M and 0.4 M KCl to give rise to 9S and 7.2S forms respectively (Fig. 4).

HPLC of the high molecular weight 11S form, in low salt conditions a single peak of radioactivity corresponding to a value for the Stokes radius of 8.7 nm. This value when combined with the sedimentation coefficients gives an estimated hydrodynamic molecular weight of 402 kDa (Table 1). Various values determined for other components are given in the same table.

Sections of the polyacryamide gel containing electrophoretically separated protein renatured in the presence of [^{3}H]R1881 with or without unlabelled R1881 were subjected to dextran-coated charcoal and the bound radioactivity measured. It was within sections separating protein having a molecular weight 100–300 kDa that displaceably bound [^{3}H]R1881 was detected. Renatured samples were separated in glycerol gradients and the displaceably bound

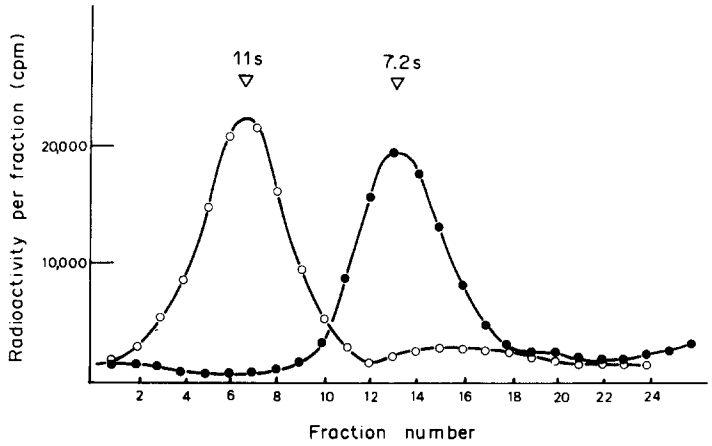

Fig. 3. Partially purified polymin extract samples were incubated overnight in the presence of [^{3}H]R1881 (5 nM) and then separated on glycerol gradient in the absence or presence of 0.4 M KCl.

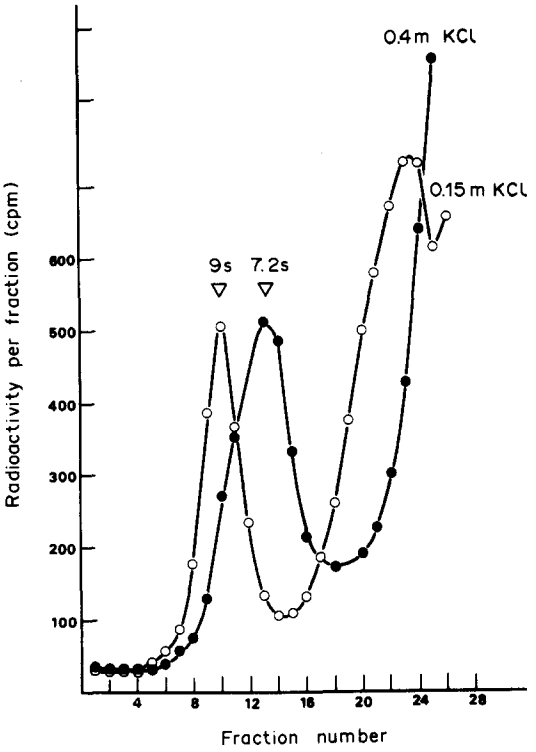

Fig. 4. The supernatant after 0.015% polymin precipitation of cytosol protein was incubated overnight with [^{3}H]R1881 (5 nM). Aliquots were then run on glycerol grandients under various ionic conditions.

[^{3}H]R1881 found to separate as a 4.5S protein. The ideal conditions for renaturation of SDS–PAGE separated protein involved a 20-fold dilution of the 6 M guanidine hydrochloride used in the initial gel extract and 1 h incubation at 0°C.

Photoaffinity labelling of partially purified receptor followed by SDS–PAGE electrophoresis (Fig. 5) confirmed that the receptor separates in the 115 kDa molecular weight range of the gel. The efficiency of photoaffinity labelling 10%) is low but the receptor concentration in the polymin extract was high the best conditions for photoaffinity labelling have not been delineated but it was found that breakdown increases with increased illumination and if too great a purity of receptor is used. However, a partial purification is required as has been reported by other workers [21].

Table 1. Molecular weights (mol. wt) estimated from sedimentation coefficients (S) and stokes radii (R_s) using the formula derived by Siegel and Monty [28] to estimate the molecular weight and fractional ratios (f/f_0) due to shape

R_s(nm)	S($\times 10^{-13}$)	Mol. wt (kDa)	(f/f_0)
8.7	11	402	1.64
8.1	9	306	1.67
7.0	7.2	213	1.64
6.0	4.5	113	1.73
3.9	3.5	58	1.39

The DHT-resin previously used for SBP purifications [20, 22] satisfactorily binds the androgen receptor, the small amount of SBP present in the cytosol extracts was removed by the partial purification routine. Little uptake of androgen receptor by the affinity resin occurred if cytosol was passed directly through the DHT-affinity column. This was found to be mainly due to the presence of estrogen receptor that also binds to the resin. This receptor was effectively removed by the partial purification procedure and whereas saturation if the cytosol receptor using diethylstilboestrol would increase the binding of androgen receptor to the resin it had no beneficial effect when added to partially purified receptor preparations. The maximum capacity of the resin for the androgen receptor was found to be 150 pmol/g.

Heparin Sepharose proved to be a good absorbant of the receptor after affinity chromatography. It was possible to wash the heparin sepharose very thoroughly without eluting bound radioactivity. Elution with 1 ml KCl (1 M) then recovers some 40% of the bound [^{3}H]R1881 but no further elution of activity was possible with salt concentrations up to 4 M KCl. However, washing with 6 M urea (1 ml) recovers all the remaining bound activity. The protein eluted with KCl or urea separates as a single band on SDS–PAGE electrophoresis (Fig. 6).

DISCUSSION

Polyethyleneimine, well known as a precipitant of proteins and nucleic acids, has a distinctive property in the uterine cytosol that it favours the formation of high molecular weight forms of the receptor. In its presence an 11S component is the only form of receptor present at low ionic strength and this transforms into 9S and 7.2S forms in the presence of increasing salt concentration. A 4.5S form predominates in the absence of polymin and this transforms into a 3.5S form in the absence of molybdate. however, the interaction between steroid binding units is not so much determined by the absence or presence of molybdate as by the salt concentration and absence or presence of ions such as Ca^{2+} and Zn^{2+} that may be chelated by EDTA [23].

Such high molecular weight complexes of the androgen receptor have been described by others, Covard and Wilson [24] propose that the

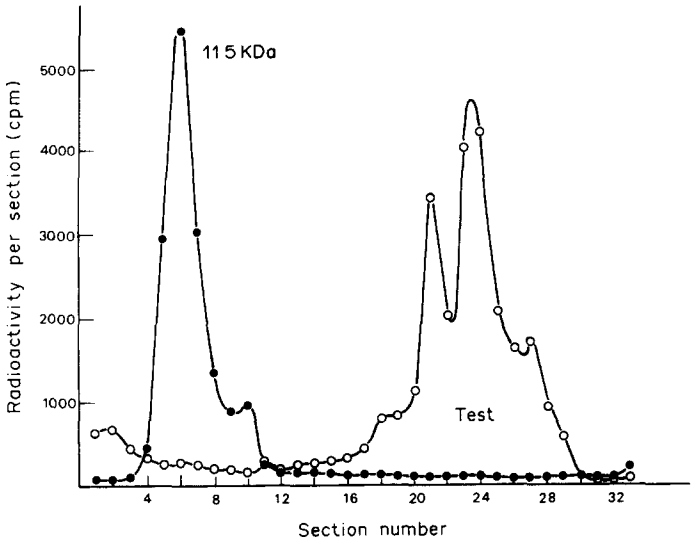

Fig. 5. Radioactivity measured in gel sections following SDS–PAGE electrophoretic separation of photoaffinity labelled samples of partially purified receptor equilibrated with [^{3}H]R1881 in the presence or absence of 1 μM testosterone. The samples were cooled to 0°C and irradiated for 2 min using a high pressure mercury lamp. The water that circulates around the lamp acts as a heat filter.

highest molecular weight form (400 kDa) represents a tetramer with two steroid binding and two non-steroid binding units. The non-steroid binding units were not identified as the 90 kDa heat shock protein so it remains a question as to whether or not the 'non steroid binding' protein is part of the active form of the receptor. Rowley *et al.* [25] have suggested that RNA is present as an important component of the 7.7S form but not present in the 9S form. Their conclusions are based on indirect evidence using RNAase and it is probable that the RNA was a minor component in their preparations, having a stabilising role but no important structural role.

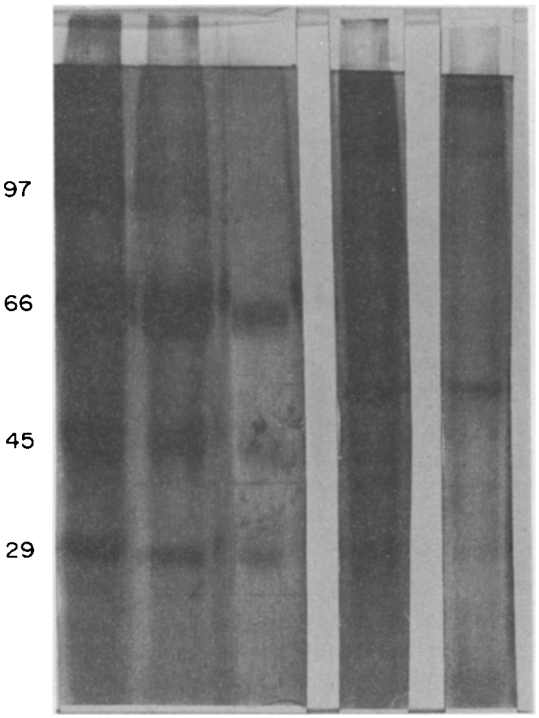

Fig. 6. Shows the photograph of a silver stained SDS–PAGE gel after separation of a 20 μl aliquot of purified receptor separated from the heparin Sepharose column in the presence of 1 M KCl, after passing DES saturated cytosol (column 4) or partially purified protein (column 5) through the affinity resin. Compared with mixed protein standards containing 2250, 750 and 150 ng total protein (columns 1–3 respectively).

The higher molecular weight forms need to be stabilised and polymin, at low concentrations, proved to be very effective. In controlled conditions they may be partially purified and studied so as to obtain better physical characteristics. Development of monoclonal antibodies against the receptor [26] will help to identify the constituent proteins in these complexes and hence determine whether or not they play an important role in the mechanism of steroid receptor actions/interactions.

Affinity labelling of the androgen receptor in rate prostate cytosol by Chang *et al.* [27] suggested a molecular weight of 86 kDa for the receptor, a value also obtained for the receptor isolated from the human preputial and cultured genital skin fibroblasts [29] and for the DNA binding form of the androgen receptor isolated from calf uterus [12]. Our results demonstrate a higher molecular weight (115 kDa) for the uterine androgen receptor that is in line with those found for the size of the androgen receptor in several other tissues [11, 16, 17]. It is generally

agreed that the monomeric receptor is unstable and in the final stages of purification the receptor breaks down into a smaller fragment about half the molecular weight of the original receptor fragment. This smaller (3.5S) steroid binding fragment would correspond to that found in the cytosol and represents the most stable form of receptor that binds both steroid and DNA [16].

Such DNA binding fragments may be considered as basic functional units of steroid hormone receptors [30] but their monomeric molecular weights are considerably larger [1, 31, 32]. The androgen receptor is one of the largest and its relative instability may be due to the presence of a long polyglycine sequence in the middle of its amino acid sequence [1]. It is possible to imagine the androgen receptor as having a dumbell structure in which the DNA/steroid binding fragment is linked by a polyglycine α helix to the transcriptionally active fragment. It thus resembles the lambda repressor and GAL4 [33, 34] which also have separate domains for DNA binding and transcription activation. Such gene regulatory proteins may bind and act cooperatively at two adjacent sites on the DNA [35], and cooperatively at a distance as the DNA wraps itself around other proteins required for gene activation [36] or nucleosomes [37, 38].

The size of the monomeric unit as predicted from hydrodynamic parameters is greater then that determined from the amino acid sequence and this difference is of the same magnitude as exists for the molecular weight of the progesterone receptor as predicted from amino acid sequence [39] or hydrodynamic properties [31]. In both instances it is expected that the receptor exists in a chemically modified form. It is therefore important that further progress is made with the purification and the physical and chemical characterisation of this receptor.

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