J. Steroid Biochem. Molec. Biol. Vol. 37, No. 5, pp. 697–705, 1990
Printed in Great Britain.

0960-0760/90 $3.00 + 0.00

SPECIFIC BINDING OF ANDROGEN AND ANDROGEN–RECEPTOR COMPLEX BY MICROSOMES FROM RAT VENTRAL PROSTATE

JAIME STEINSAPIR* and THOMAS G. MULDOON
Department of Physiology and Endocrinology, Medical College of Georgia, Augusta, GA 30912, U.S.A.

(Received 17 April 1990)

Summary—Microsomes from rat ventral prostate show the presence of a high affinity–low capacity population of androgen-binding sites with affinity for ionic exchange resin similar to that of cytosol androgen receptor (AR), as manifested by similar results obtained with hydroxylapatite. The affinity for mibolerone was similar for both forms ($K_a = 0.5$–$2.9 \times 10^{10}\,M^{-1}$). The membrane-bound form can be extracted in hypotonic buffer, with retention of binding properties. Isotonic sucrose allowed higher degree of extractability of the microsomal AR than 10% (v/v) glycerol. The presence of hormone lends stability to the microsomal AR, while high salt or nonionic detergents have a deleterious effect on their longevity. The microsomal receptor form is not sensitive to serine-proteases as opposed to the cytosol AR. After exhaustive extraction of binding sites, microsomes are capable of accepting cytosol mibolerone–receptor complexes to a level corresponding to the concentration of depleted binding sites; microsomes from non-target tissue do not manifest such capability. Microsomal AR complexes do not bind DNA and they are not activated after heat treatment. Mixed preparations of extracted microsomal complexes with cytosol complexes showed heat-induced increased ability to bind DNA to the same level of diluted cytosol complex alone, indicating the absence of a microsomal inhibitor of DNA binding. The results indicate the co-existence of a non-DNA binding form of the AR in the microsomal membranes with the classical DNA binding form of the AR present in the cytosol of ventral prostate homogenates.

INTRODUCTION

Early studies on the subcellular distribution of steroid receptors have indicated that most of the receptor resides in the nucleus. The cytosol receptor could be a loosely bound chromatin form of the receptor that leaks during homogenization and tissue fragmentation [1]. Previous studies concerning total cellular dynamics of steroids and steroid receptor turnover have also recognized transient docking of radiolabelled steroid–receptor complexes within the microsomes [2]. These microsomal sites may have been overlooked due to the low resolution of immunocytochemical studies at the light microscope level using monoclonal antibodies to receptor protein [3]. When electron microscopy is used, ultrastructural localization of steroid receptor indicates its physical presence in both nuclear and microsomal compartments of target cells for steroids [4]. Furthermore, the techniques used by Welshons *et al.* [1] to produce nucleoplasts, with which most of the cell's steroid receptor is associated, result in production of nuclei with cytoplasmic attachments, specially membranes, and the association of steroid receptor with membranes would give the appearance of nuclear association of the receptor. Little *et al.* [5–7] have been able to solubilize the microsomal estrogen receptor from pig uterus by the use of low ionic strength phosphate buffers. However, the interpretation of the results has been hampered due to the absence of proper conditions to minimize receptor breakdown and increase stability of the binding sites [5–7]. A microsomal androgen receptor (AR) has also been described in rat ventral prostate and its binding properties seem different from the cytosolic AR [8]. However, no attempts have been done to solubilize the microsomal AR. We examine here the binding properties of extracted microsomal AR, under conditions that maximize stability of the binding sites.

*To whom correspondence should be addressed.

EXPERIMENTAL

[17α - methyl - ^{3}H]Mibolerone(7α,17α-dimethyl-19-[17α-methyl-^{3}H]nortestosterone;

88 Ci/mmol) and radioinert mibolerone were purchased from Amersham, Searle. Dextran-coated charcoal (DCC) was prepared with Norit A (0.5%, Amend Drug and Chemical Co., Irvington, N.J.) and Dextran (0.05%; Nutritional Biochemistry Co., Cleveland, Ohio). Hydroxylapatite (Bio-gel HTP) was from Bio-Rad (Richmond, Calif.). DNA–cellulose (double stranded, from calf thymus), cellulose, Triton X100 and sodium cholate were from Sigma. All other reagents were of analytical grade.

Tissue preparation and subcellular fractionation

Male rats were obtained at 120 days of age (400–440 g body wt) from Holtzman (Madison, Wisc.) and allowed a 5-day period of acclimatization to a 14–10 h light–dark cycle at a temperature of 72°F. Rats were fed with lab chow (Wayne) and tap water *ad libitum*. Bilateral orchidectomy was performed in all cases through the scrotal route under ether anesthesia 24 h before each experiment. Animals were sacrificed by decapitation and individual ventral prostates were collected in ice-cold homogenization buffer (TEDMSP) containing 10 mM Tris base, 1.5 mM disodium EDTA, 0.5 mM dithiothreitol, 10 mM sodium molybdate, 8.5% (w/w) sucrose and 1 mM phenylmethylsulfonylfluoride (PMSF), pH 7.4 at 4°C, rinsed and minced with scissors prior to homogenization by three 15-s bursts and 30-s cooling periods, with a Polytron PT10 homogenizer (set 4) at a (tissue/buffer) ratio of 550 mg/ml (1 ml/prostate). The homogenate was centrifuged at 800 ***g*** for 20 min. The supernatant (cytoplasm) was centrifuged at 11,000 ***g*** for 15 min and the postmitochondrial supernatant was then centrifuged at 223,000 ***g*** for 60 min to yield the cytosol and the microsomal pellet. Microsomal pellets were rinsed with 1 ml of homogenization buffer to remove cytoplasmic contaminants and resuspended in this buffer or alternatively washed and extracted by resuspension at 550 mg/ml in homogenization buffer, centrifugation at 223,000 ***g*** for 1 h and reconstitution at the same (tissue/buffer) ratio. Microsomal membrane suspensions were gently rehomogenized by 3 or 4 strokes of a Dounce homogenizer (pestle A) to yield the source of the microsomal binding activity.

Binding assays

Isolated subcellular fractions (microsomes, cytosol) from 24 h castrated rat ventral prostate homogenates were used as source of saturation binding analysis. Equilibrium association binding constants (K_a) and concentration of binding sites (B_{max}) were determined after separation of bound and unbound steroid and Scatchard analysis of the data [9].

Microsomal binding assay

Microsomal suspensions were analyzed for specific androgen binding properties by incubation of samples (250 μl) for 20 h at 4°C in the presence of (0.05–2.5 nM) [^{3}H]-mibolerone with or without 5–250 nM radioinert mibolerone (250 μl/tube, final volume: 1 ml). Bound and unbound mibolerone were separated by dextran-coated charcoal (DCC) adsorption of free and loosely bound steroid. 1 ml of dextran-coated charcoal was added, incubation was performed for 10 min at 4°C and the tubes were centrifuged at 860 ***g*** for 10 min. The supernatants were collected in scintillation vials; 10 ml of Permablend (Packard) was added per vial. Samples were shaken for 2 h and counted. Hydroxylapatite was also used and compared with dextran-coated charcoal in both microsomal and cytosolic fractions. The preparation and use of hydroxylapatite as a "batch" assay was described before [10].

When microsomal androgen–receptor complexes were used for DNA binding analyses, a modification of the previous method of charcoal treatment was used. Charcoal-dextran pellets were prepared from the same volume of DCC suspension as the sample volume to be charcoal-treated. This procedure avoids further dilution of the previously formed steroid–receptor complex. Charcoal pellets and samples were mixed, incubated for 10 min at 4°C and then centrifuged at 860 ***g*** for 10 min. Supernatants were decanted and collected in scintillation vials; 10 ml of Permablend (Packard) were used per vial.

Cytosol binding assay

Cytosol was removed using previously chilled Pasteur pipettes and 250 μl aliquots were subsequently incubated in a total volume of 1 ml with increasing concentrations (0.05–2.5 nM) of ^{3}H-labelled ligand. Identical samples were incubated with a 100-fold excess of radioinert mibolerone for determination of non-specifically bound ^{3}H-labelled ligand. Cytosol fractions were incubated at 4°C for 20 h. The separation of bound and unbound steroid was accomplished by hydroxylapatite adsorption of the steroid–receptor complex [11]. The hydroxyl-

apatite suspension was prepared exactly as previously described [10].

The effects of molybdate and PMSF in the microsomal and cytosol androgen receptor levels were studied by selective deletion of each component from the homogenization buffer. Subsequent isolation of cellular fractions and saturation binding analysis was performed as described before.

Binding properties of the extracted microsomal androgen receptor

We compared first the binding properties of unextracted and extracted microsomal AR of ventral prostate. Furthermore, microsomes were prepared in homogenization buffer, in the presence of 8.5% (w/w) sucrose or 10% (v/v) glycerol in order to compare their ability to extract microsomal AR. Different extraction periods (2 or 19 h) were examined, and centrifugation at 223,000 ***g*** for 60 min allowed the analysis of binding in both the extracted and residual material. Saturation binding analyses for additional 18 h at 4°C was performed and dextran-coated charcoal used to remove unbound steroid.

Extraction was also performed in the presence of saturating concentrations (5 nM) of [^{3}H]mibolerone with or without a 100-fold molar excess of radioinert mibolerone, using the same extraction periods and procedure previously selected for the study of extractability of unbound AR.

The use of KCl was applied to further examine the degree of extractability of the androgen binding sites from the microsomal framework. Hypotonic (0.05 M), isotonic (0.15 M) and hypertonic (0.40 and 0.60 M) KCl concentrations were used. The postmitochondrial supernatants were split into equal aliquots in order to obtain the same starting amount of microsomal protein. Microsomal pellets were exposed to different KCl concentrations prepared in homogenization buffer (TEDMSP), extracted for 2 or 19 h at 4°C and then centrifuged at 223,000 ***g*** for 60 min. Saturation binding analysis was performed in each extracted or residual material, in TEDMSP.

The sensitivity of microsomal AR to detergents of very low critical micellar concentration (Triton X100, hard to remove) or very high critical micellar concentration (sodium cholate, easy to remove) was examined using 0.01, 0.05, 0.15 and 2.0% (v/v) of each detergent [12]. Microsomes were pre-incubated for 30 min at 4°C in the absence or presence of detergent. The microsomal membranes were subsequently washed three times with ten times the volume of buffer to remove the detergent. The final pellets were reconstituted in known volumes of TEDMSP and examined for specific androgen binding activity using saturation binding analyses, as previously described, in each condition. Control membranes were treated similarly without the detergent.

Acceptor capability of cytosol androgen–receptor complexes by microsomes from ventral prostate

Since it has been possible to show that microsomes can be "exhausted" of their high affinity estrogen-binding proteins in other systems [13] and that such exhausted microsomes could function as acceptors for cytosol receptor, a similar approach was undertaken to examine whether the microsomal membranes could have such properties in the prostate androgen receptor system.

Prostate microsome pellets were prepared and aliquots were taken to measure control binding levels by saturation analyses. The pellets were extracted by centrifugation at 223,000 ***g*** for 60 min and constituted the "exhausted" microsomal preparation. Aliquots were used to analyse by saturation binding analyses the remaining specific binding. The "exhausted" resuspended preparations were also used for mixing with an equivalent volume, 3 or 4 vol of original cytosol; these samples represented reconstituted preparations. After mixing and centrifugation at 223,000 ***g*** for 60 min, the reisolated membranes were resuspended and subjected to saturation binding analyses. Dextran-coated charcoal was used to remove unbound steroid for all binding analyses.

DNA binding ability of microsomal and cytosol androgen receptor complexes

In an effort to explore other putative properties of the microsomal androgen receptor, we examined the DNA binding ability of these moieties as compared to the typical cytosolic form of the receptor.

Microsomes or cytosol were labelled with 5 nM [^{3}H]mibolerone during 20 h at 4°C. Unbound steroid was removed with DCC and microsomal complexes were extracted by centrifugation at 223,000 ***g*** for 1 h. Heat-attempted activation of hormone–receptor complexes was performed by incubation of appropriate fractions at 24°C for 30 min and subsequent cooling

at 4°C for an additional 30 min. Aliquots (400 μl) were then incubated for 45 min at 4°C with 250 μl of packed DNA–cellulose (range: 8–125 μg DNA/tube) in a final volume of 1 ml with homogenization buffer, in duplicate. Samples were vortex-mixed at 15 min intervals and then washed three times with 3 ml of homogenization buffer at 860 *g* for 15 min each time. The final supernatants were discarded and the final pellets were extracted twice with 1 ml ethanol at 30°C for 20 min. Pooled ethanol extracts obtained by centrifugation at 800 *g* for 10 min were counted for radioactivity and the pellets were used for DNA determinations [14]. DNA–cellulose and cellulose alone were mixed in varying proportions so that the cellulose content was kept constant in each tube.

DNA–cellulose and cellulose pellets were washed twice with 3 ml of homogenization buffer supplemented with 1.5 mM $MgCl_2 \times 6H_2O$, pH 7.4 (22°C) and usually frozen (−20°C) for 24 h until use. Incubation of DNA with increasing amounts of nonpurified receptor complexes leads to an artifactual saturation [15, 16]. This problem can be solved by incubating a constant amount of steroid–receptor complex (binder) with varying amounts of DNA (ligand), as it was previously described in the estrogen receptor system [15]. Radioactivity bound to DNA represented the amount of androgen–receptor complex specifically bound to DNA–cellulose when corrected for binding of complexes to cellulose alone in the presence or absence of radioinert hormone. Control samples were also prepared in the presence of saturating concentrations of [^{3}H]mibolerone, with or without a 100-fold molar excess radioinert mibolerone. There was no specific binding of the labelled androgen alone to DNA–cellulose under these receptor-free conditions.

Analytical procedures

Radioactivity was quantified with a Beckman LS-7500 spectrometer with built-in capacity for DPM conversion. Efficiency for tritium was 56%. Microsomal samples were counted in a mixture composed of 5 g of Permablend II (Packard) dissolved in 1 l of toluene. Cytosolic samples were counted in a mixture composed of 15 g of butyl-PBD (2-(4′-tert-butylphenyl)-5-(4′-biphenylyl)1,3,4-oxadiazole; Sigma), 1770 ml toluene, 620 ml S-570 (ethoxylated octylphenols, Fisher) and 110 ml distilled water. Counting was done at a level permitting <2% error. The method of Lowry *et al.* [17] was used for determination of protein. Aliquots of detergent-treated membrane samples were perchloroacetic-precipitated and ether–ethanol (1:3)-extracted to measure protein according to Torngvist and Belfrage [18]. The method of Burton [14] was used for determination of DNA.

RESULTS

Initial analysis of the properties of microsomal androgen binding sites present in ventral prostate was performed using two different methods of separation of bound and unbound steroid. There were no differences in number of sites as well as in the affinity of mibolerone for either charcoal or hydroxylapatite (data not shown). Differential sensitivity of the binding proteins to protective agents was examined by selective changes of composition of the homogenization buffer. Cytosol AR but not microsomal AR was sensitive to serine-proteases, as manifested by a decrease in the number of cytosol binding sites in the absence of PMSF (data not shown). The absence of molybdate decreased both number and affinity of microsomal and cytosol androgen binding sites for mibolerone (data not shown). We subsequently compared the binding properties of unextracted and extracted microsomal AR. Both preparations manifested similar equilibrium binding properties (Fig. 1). Extracted microsomal binding sites have the same affinity for mibolerone as the unextracted microsomal sites. The association constants for the mibolerone–receptor interaction ranged between 0.6–1.4 ($\times 10^{10}\ M^{-1}$) for the unextracted, extracted and residual membrane preparations, in two different experiments, under these conditions. There was an appreciable enrichment of the specific binding activity of the extracted receptor form, since 71% of the total microsomal protein remains in the residual material after extraction (Fig. 1). Sucrose was consistently more effective than glycerol in promoting extraction of the microsomal AR in the presence of the hormone (data not shown). The presence of mibolerone allowed full recovery of binding sites when total binding capacities of (extracted + residual) fractions are compared in the presence vs absence of ligand, regardless of the duration of extraction (Table 1).

Since it was reported before in the estrogen receptor system that microsomal binders are

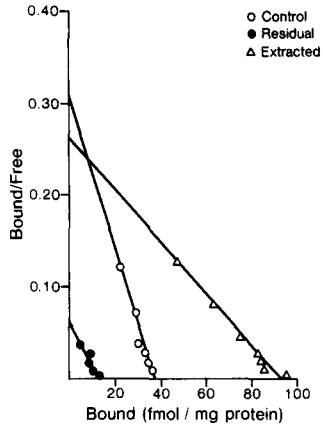

Fig. 1. Retention of equilibrium binding properties after extraction of microsomal AR. Isolated microsomes from ventral prostate were extracted or not (control, unextracted) in TEDMSP and incubated in the absence of hormone, in the same buffer, for 2 h at 4°C. Membranes were then centrifuged at 223,000 *g* for 1 h and extracted and residual fractions were subsequently assayed by saturation binding analyses. B_{max} (fmol/mg protein) were: control (○), 36.1; residual (●), 11.8; extracted (△) 92.1. K_d ($\times 10^{10}\,M^{-1}$) were: control (○), 0.9; residual (●), 0.7; extracted (△), 1.2. Protein (mg/tube) = control (○), 1.0; residual (●), 0.71; extracted (△), 0.24. Total specific binding capacities (fmol): 876 (control), 506 (extracted) and 212 (residual). Total protein (mg): 25.1 (control), 17.8 (residual) and 5.5 (extracted).

readily extractable with KCl-free hypotonic buffer or with 0.4 M KCl but are resistant to extraction with 0.15 M KCl [13] in the uterus, salt extractability of microsomal AR was examined in rat ventral prostate. Salt treatment rendered the microsomal AR unstable and induces a concentration-dependent decrease in total specific binding capacity when extracted membranes are subsequently assayed for binding activity (data not shown). When high critical micellar concentration (CMC) and low CMC detergents, namely sodium cholate and Triton X100, were used to extract microsomal AR, both detergents caused a decrease in total specific binding capacity of the microsomal AR (data not shown).

Previous findings had indicated that microsomal estrogen receptors were able to serve as acceptors for cytosol receptors after removal of microsomal estrogen-binding proteins [13]. We examined whether residual prostate microsomes had the same properties. After extraction of microsomal androgen binding proteins in homogenization buffer supplemented with isotonic sucrose, residual microsomal membranes were exposed to increasing amounts of cytosol complexes (Table 2). The concentration of specific binding sites as well as the total specific binding capacity of the mixed preparations reached levels similar to that originally seen in the microsomes, regardless of the amount of receptor presented to them (Table 2). This titration experiment revealed that the concentration of acceptor sites closely approximate the number of sites originally accepted by the microsomal AR. Similar experiments were performed using microsomal preparations from diaphragm tissue of 1-day castrated adult male rats. Washing these microsomes in a manner identical to that performed on prostate microsomes did not endow the non-target microsomes with acceptor capability for prostate cytosol–AR complex (data not shown).

Testosterone has been shown to induce a decrease in microsomal receptor levels of ventral prostate 1 h after administration [10]. This phenomenon suggested to us that besides its capacity to accept cytoplasmic steroid–receptor complex, the microsomal receptor could translocate into the nuclear compartment. It was then of interest to examine the DNA binding ability of extracted microsomal receptors as compared to that of cytosol–AR complexes before and after heat-attempted activation.

Table 1. Effect of the presence of the ligand on the extractability of microsomal androgen receptors of rat ventral prostate

Duration of extraction	% Recovery of total specific microsomal androgen-binding capacity			
	Extracted		Residual	
	− Mibolerone	+ Mibolerone	− Mibolerone	+ Mibolerone
2 h	56 ± 3	76	23 ± 2	36
19 h	53 ± 2	64 ± 3	16 ± 4	43 ± 5

Microsomal fractions were isolated after homogenization in TEDMSP and incubated in the presence or absence of mibolerone for 2 or 19 h, at 4°C. Extracted and residual fractions were obtained by centrifugation at 223,000 *g* for 1 h and assayed for binding activity as described in "Materials and Methods". KCl was not used for microsomal androgen receptor extraction. Starting total specific binding capacities of control (unextracted) groups were 0.8 ± 0.1 pmol (− mibolerone) and 1.9 ± 1.0 pmol (+ mibolerone). Values are averages ± range ($n = 2$), except 2 h (+ mibolerone) where $n = 1$.

Table 2. Acceptor capability of microsomes for cytosol receptors after removal of microsomal androgen receptor

Condition	Concentration of specific binding sites (fmol/mg protein)	K_a (10^{10} M^{-1})	Total specific binding capacity (pmol)
Original	49 ± 7	4.5 ± 2.2	0.33 ± 0.04
Exhausted	20 ± 4	2.0 ± 0.3	0.11 ± 0.03
Reconstituted (1:1)	27 ± 4	3.2 ± 1.6	0.14 ± 0.02
Reconstituted (1:3)	37 ± 4	2.6 ± 1.2	0.25 ± 0.02
Reconstituted (1:4)	43 ± 9	2.9 ± 0.7	0.33 ± 0.07

Microsomes were prepared by differential centrifugation, washed and suspended in homogenization buffer with 8.5% (w/w) sucrose. One-third was taken for measurement of the "Original" specific androgen-binding capacity by saturation binding analyses. The remainder was extracted into steroid-free homogenization buffer and the pellet, representing "Exhausted" microsomes was re-isolated by ultracentrifugation at 223,000 *g* for 60 min. Half of these resuspended pellets were subjected to saturation binding analyses, and the other half was mixed with either an equivalent volume (1:1), 3 volumes (1:3) or 4 volumes (1:4) of original cytosol; these samples represented the "Reconstituted" microsomal preparations. Following mixing, the cytosol-microsome samples were subjected to saturation binding analyses after ultracentrifugation at 223,000 *g* for 60 min and use of the pelleted resuspended material as binding source. All saturation binding analyses of "original", "exhausted" and "reconstituted" preparations were performed for 18 h at 4°C. Total binding capacities were estimated from the specific binding activities and total protein content of each fraction. Results are averages ± range of two different experiments.

Figure 2 (panel A) shows that extracted microsomal receptor does not bind DNA and it is not activated after incubation at 24°C for 30 min and subsequent cooling at 4°C for an additional 30 min. These results are in agreement with absence of DNA binding ability of unextracted microsomal AR complexes (data not shown). Non activated cytosol AR complexes have measurable affinity for DNA–cellulose in the presence of molybdate and they can be activated by heat under these conditions (Fig. 3).

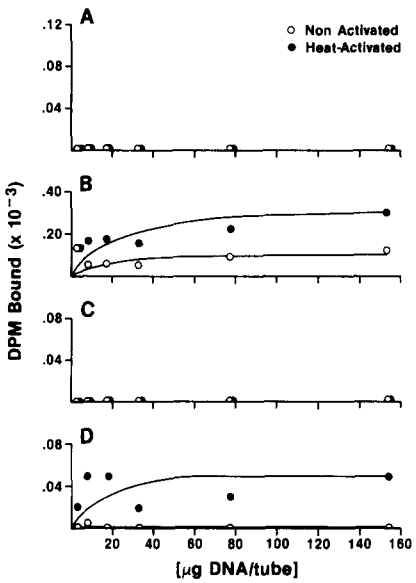

Fig. 2. DNA binding ability of microsomal androgen receptor. Microsomal pellets were labeled with 5 nM [^{3}H]mibolerone for 20 h at 4°C, unbound steroid removed with DCC and extracted by centrifugation at 223,000 *g* by 1 h ("extracted" microsomal receptor) in TEDMSP. Alternatively, microsomes were washed once by resuspension in TEDMSP and centrifugation at 223,000 *g* by 1 h and then labeled, DCC treated and extracted as before ("extracted and washed" microsomal receptor). Labeling and treatment of cytosol was the same as for microsomes.

Equimolar amounts of microsomal and cytosol complexes were mixed or not and after heat-attempted activation (24°, 30 min). DNA binding assays were performed as described in "Materials and Methods". Inputs: 31.2 fmol/tube for "extracted" microsomal receptor alone (panel A) or in the presence of equal amount of cytosol receptor (panel B) and 12.1 fmol/tube for "extracted and washed" microsomal receptor alone (panel C) or in the presence of equal amount of cytosol receptor (panel D). The absence of DNA binding ability of microsomal complexes was observed in three different experiments.

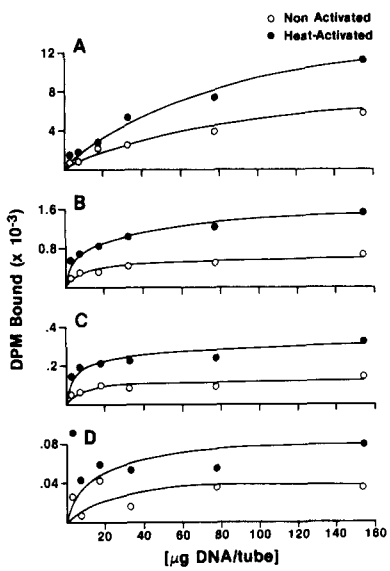

Fig. 3. DNA binding ability of cytosol androgen receptor. Cytosol of 24 h castrated rat ventral prostate was isolated, labeled with 5 nM [^{3}H]mibolerone for 20 h at 4°C, unbound steroid removed with DCC and then diluted with homogenization buffer to the following levels of input per tube: 952 (panel A), 84.2 (panel B), 22.1 (panel C) and 10.1 (panel D) fmol. After heat-attempted activation (24°C, 30 min) of appropriate aliquots, DNA binding assays were performed as stated in "Materials and Methods". Increased DNA binding ability of cytosol complexes after heat treatment was observed in three different experiments.

The presence of an inhibitor of binding of microsomal–AR complexes to DNA which could have explained the lack of activation by heat treatment of the microsomal AR was examined by mixing experiments. Figure 2 (panel B) shows the results of mixing equimolar amounts of extracted microsomal complexes with cytosol–receptor complex. Previously washed microsomal membranes were also labelled, extracted and mixed or not in equimolar amounts with cytosol complexes (Fig. 2, panels C and D). Both approaches showed absence of activation of the microsomal complexes (Fig 2, panels A and C) alone and appearance of increased DNA binding ability of the mixed preparations after heat-attempted activation (Fig. 2, panels B and D), to a level similar to appropriate controls performed with different dilutions of cytosol complexes alone (Fig. 3). These results indicate the absence of an inhibitor of DNA binding of microsomal complexes and the co-existence of a non-DNA binding form of the AR in the microsomes with the typical DNA binding form of the AR present in the cytosol of prostate homogenates.

DISCUSSION

Microsomal binding sites for steroids have been described in a variety of tissues: estradiol binding sites in rat liver [19], pig uterus [5, 6], and calf uterus [20], progesterone binding sites in rat liver [21], dexamethasone binding sites in rat liver [22, 23] and androgen binding sites in rat ventral prostate [24]. Conditions for salt extractability of the microsomal receptors fluctuate in different systems: low ionic strength phosphate buffers are necessary in pig uterus [7], and high KCl (0.4–0.6 M KCl) in rat uterus [13, 25] for the estrogen receptor form. The results reported here suggest that KCl does not "unmask" additional microsomal androgen binding sites of ventral prostate and in fact, is deleterious to the binding site, decreasing the total microsomal androgen binding capacity and increasing the dissociation constant of mibolerone from the receptor. The use of detergents has also been applied for solubilization of microsomal steroid receptors. Detergent extraction of uterine microsomes has been attempted with Lubrol-PX and cholate, which caused a decrease in specific-binding of estradiol in the uterine system [20]. However, Triton X100 and Nonidet P-40, at concentrations of 0.05–0.5%, increased estrogen binding, apparently due to selective removal of non-receptor protein, (the recovery of microsomal protein decreased) and uncovering of previously buried binding sites. Losses in microsomal binding capacity observed here with sodium cholate and Triton X100 can be explained by removal of the microsomal androphiles. Alternatively, the decrease in binding activity observed even with low concentration of both detergents can be explained by protein denaturation of a membrane-bound receptor which subsequently would increase ligand dissociability. The microsomal AR can be extracted from the membrane framework by the use of hypotonic (+ glycerol) or isotonic (+ sucrose) buffers, in the absence of salt or detergents, with retention of binding properties, under conditions that minimize receptor breakdown. The stability of the extracted microsomal androgen binding sites was further increased by the presence of androgen.

Differential properties of the microsomal and cytosol androgen receptor forms were manifested by the study of the effects of protective agents in receptor levels. Absence of protease sensitivity of microsomal steroid receptors has been shown before in the glucocorticoid receptor system [23], as opposed to the high sensitivity to proteolytic activity of the cytosol steroid receptors [26, 27]. These results are in agreement with those reported here. The binding sites for both microsomal and cytosol androgen receptors could have disulfide bonds important for hormone binding, given the sensitivity of both androphiles to the presence of molybdate. The absence of molybdate could promote disassembly of the tetrameric structure of the androgen receptor, with subsequent subunit dissociation by reduction of key sulfhydryl groups needed for interaction with the hormone. The existence of a finite number of sites within the endoplasmic reticulum able to recognize cytosol receptor protein and bind them was tested by extraction of microsomal receptors from the membranes with isotonic buffer and mixing of receptor-extracted microsomes with cytosol. Addition of cytosol receptor in various amounts led to a binding of receptor to the microsomes to a level similar to that originally seen before extraction, but not higher. The structure of the endoplasmic reticulum membrane may have therefore two distinct sites of interaction with steroid hormone: the receptor itself and on the other hand, acceptor sites able to bind steroid hormone–receptor complex, but not hormone alone. Microsomal protein or

RNA are two obvious candidates as critical components of these acceptor sites, which are tissue-specific for target cells. Specificity of the interaction of receptor complexes with target membranes could be dictated by the structure of the membrane and/or that of the receptor complex. Microsomal receptors may modulate nuclear receptor turnover and recycling by virtue of producing acceptor sites for uptake of free or loosely associated nuclear complexes, previously thought to exist in the cytosol.

Activation of steroid receptors to a form having enhanced DNA-binding properties is an acknowledged crucial event in the mechanism of steroid hormone action and this activation can be effected *in vitro* by exposure to controlled heating. The present studies demonstrate failure of microsomal receptors to bind DNA, or to acquire DNA-binding capability following heat-attempted activation. The non-activated cytosol receptors manifested ability to bind DNA in the presence of molybdate. Heat-induced activation increased the number of receptor complexes bound to DNA and molybdate did not prevent heat activation. Heat may alter the receptor structure in such a way as to expose masked DNA-binding sites [28–30]. The observation that microsomal receptors have no affinity for DNA, coupled with the finding that such binding activity cannot be elicited by heat treatment, suggests that these moieties are not directly involved in the gene regulation phase of androgen action. A comparison was subsequently made between the DNA binding abilities of solubilized microsomal androgen receptors, mixed preparations of equimolar amounts of microsomal and cytosol–receptor complexes, and cytosol complexes alone. The results indicate absence of cytosol receptor contamination of the microsomal preparations since cytosol receptor presence would have been manifested by increased DNA-binding ability of the extracted microsomal receptor preparation alone, after heat treatment. Furthermore, mixed preparations of extracted microsomal complexes with cytosol complexes did not display increased levels of activation as compared to the degree of activation of similar dilutions of cytosol complexes alone. Alternatively, the lack of DNA-binding ability of microsomal androgen receptor complexes could have been explained by the presence of an inhibitor of DNA binding of the membrane-bound complexes. However, the absence of inhibition of heat-induced increase in DNA binding ability of cytosol receptor complexes after mixing with equimolar amounts of extracted microsomal complexes indicate the absence of such inhibitory factor. The overall results suggest the presence of a non DNA-binding form of the androgen receptor associated with the microsomes. The ready extractability of the microsomes by androgens and the absence of DNA-binding ability of the microsomal complexes suggests that the complexes may become mobilized for activity at distant points within the cell, perhaps involving extranuclear actions of steroid hormones.

Acknowledgement—This work was supported by grant DK32046 from the NIDDK, NIH.

REFERENCES

1. Welshons W. V., Lieberman M. E. and Gorski J.: Nuclear localization of unoccupied estrogen receptors. *Nature* **307** (1984) 747–749.
2. Cidlowski J. A. and Muldoon T. G.: The dynamics of intracellular estrogen receptor regulation as influenced by 17β-estradiol. *Biol. Reprod.* **18** (1978) 234–246.
3. King W. J. and Greene G. L.: Monoclonal antibodies localize oestrogen receptor in nuclei of target cells. *Nature* **307** (1984) 745–747.
4. Perrot-Aplanat M., Groyer-Picard M. T., Logeat F. and Milgrom E.: Ultrastructural localization of the progesterone receptor by an immunogold method: effect of hormone administration. *J. Cell Biol.* **102** (1986) 1191–1199.
5. Little M., Rosenfeld G. C. and Jungblut P. W.: Cytoplasmic estradiol "receptors" associated with "microsomal" fraction of pig uterus. *Z. Physiol. Chem.* **353** (1972) 231–242.
6. Little M., Szendro P. I. and Jungblut P. W.: Hormone-mediated dimerization of microsomal estradiol receptor. *Z. Physiol. Chem.* **354** (1973) 1599–1610.
7. Little M., Szendro P. I., Teran O., Hughes A. and Jungblut P. W.: Biosynthesis and transformation of microsomal and cytosol estradiol receptors. *J. Steroid Biochem.* **6** (1975) 493–500.
8. Steinsapir J., Bryhan M. and Muldoon T. G.: Relative binding properties of microsomal and cytosolic androgen receptor species of the ventral prostate. *Endocrinology* **125** (1989) 2297–2311.
9. Scatchard G.: The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **11** (1949) 660–672.
10. Steinsapir J., Evans A. C. Jr, Bryhan M. and Muldoon T. G.: Androgen receptor dynamics in the rat ventral prostate. *Biochem. Biophys. Acta* **842** (1985) 1–11.
11. Pavlik E. J. and Coulson P. B.: Hydroxylapatite "batch" assay for estrogen receptors: increased sensitivity over present receptor assays. *J. Steroid Biochem.* **7** (1976) 357–368.
12. Helenius A., McCaslin D. R., Fries E. and Tanford C.: Properties of detergents. In *Methods in Enzymology, Biomembranes* (Edited by S. Fleischer and L. Parker). Academic Press, New York, Vol. LVI (1979) pp. 734–739.
13. Watson G. H. and Muldoon T. G.: Specific binding of estrogen and estrogen–receptor complex by microsomes from estrogen-responsive tissues of the rat. *Endocrinology* **117** (1985) 1341–1349.
14. Burton K.: A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62** (1956) 315–323.

15. Baskevitch P. P., Vignon F., Bousquet C. and Rochefort H.: Increased DNA binding of the estrogen receptor in an estrogen-resistant mammary cancer. *Cancer Res.* **43** (1983) 2290–2297.
16. Andre J. and Rochefort H.: *In vitro* binding of the estrogen receptor to DNA: absence of saturation at equilibrium. *FEBS Lett.* **50** (1975) 319–323.
17. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
18. Torngvist H. and Belfrage P.: Determination of protein in adipose tissue extracts. *J. Lipid Res.* **17** (1976) 542–545.
19. Blyth C. A., Cooper M. B., Roobel A. and Rabin B. R.: The binding of steroid hormones to degranulated microsomes from rat-liver endoplasmic reticulum. *Eur. J. Biochem.* **29** (1972) 293–300.
20. Parikh I., Anderson W. L. and Neame P.: Identification of high affinity estrogen binding sites in calf uterine microsomal membranes. *J. Biol. Chem.* **255** (1980) 10266–10270.
21. Drangova R. and Feuer G.: Progesterone binding by the hepatic endoplasmic reticulum of the female rat. *J. Steroid Biochem.* **13** (1980) 629–637.
22. Omrani G. R., Furukawa H., Sherwood J. A. and Loeb J. N.: [^{3}H]-dexamethasone binding by rat liver microsomes: effects of age, sex and adrenal status. *Endocrinology* **112** (1983) 178–186.
23. Ambellan E., Swanson M. and Davidson A.: Glucocorticoid binding to rat liver microsomal fractions *in vitro*. *J. Steroid Biochem.* **14** (1981) 421–428.
24. Robel P., Blondeau J. P. and Baulieu E.-E.: Androgen receptors in rat ventral prostate microsomes. *Biochem. Biophys. Acta* **373** (1974) 1–9.
25. Labate M. E., Whelly S. M. and Barker K. L.: Ribosome-associated estradiol-binding components in the uterus and their relationship to the translational capacity of uterine ribosomes. *Endocrinology* **119** (1986) 140–151.
26. Wilson E. M. and French F. S.: Effects of proteases and protease inhibitors on the 4.5S and 8S androgen receptor. *J. Biol. Chem.* **254** (1979) 6310–6319.
27. Sherman M. R., Pickering L. A., Rollwagen F. M. and Miller L. K.: Meroreceptors: proteolytic fragments of receptors containing the steroid-binding site. *Fedn Proc.* **37** (1978) 167–173.
28. Milgrom E., Atger M. and Baulieu E.-E.: Acidophilic activation of steroid hormone receptors. *Biochemistry* **12** (1978) 5198–5205.
29. Becker P. B., Gloss B., Schmid W., Strahle V. and Schutz G.: *In vivo* protein-DNA interactions in a glucocorticoid response element require the presence of the hormone. *Nature* **324** (1986) 686–688.
30. Wellman T. and Beato M.: Steroid-free glucocorticoid receptor binds specifically to mouse mammary tumour virus DNA. *Nature* **324** (1986) 688–691.