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RESOLUTION OF NON-ACTIVATED AND ACTIVATED ANDROGEN RECEPTORS BASED ON DIFFERENCES IN THEIR HYDRODYNAMIC PROPERTIES

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Summary—This study shows that cytosolic androgen receptor of rat ventral prostate sediments at 10–11 S on conventional low salt sucrose density gradients (SDG), and at 4.6 S on high salt SDG, whether it is activated or not; inclusion of 10 mM Na_2MoO_4 in all buffers does not alter these sedimentation coefficients. In the presence of 50 mM Na_2MoO_4 non-activated and activated androgen receptors sediment in high salt SDG at 7–8 S and 4.6 S, respectively. Thus the presence of high concentrations of molybdate during centrifugation inhibits the KCl induced disaggregation of receptor into subunits. Similar effects are observed on Sephacryl-S200 gel filtration; in 50 mM MoO_4^{2-} and 0.4 M KCl non-activated receptor has an estimated Stokes radius of 67 Å; this value decreases to 52 Å upon activation in the presence of proteolysis inhibitors; omission of molybdate during chromatography yielded 52 Å and 27 Å entities. Estimated mol. wts are 198,000 Daltons for the non-activated 67 Å form and 98,000 Daltons for the activated 52 Å receptor. Sodium molybdate (50 mM) prevents temperature (18°C) and high ionic strength (0.4 M KCl) induced receptor activation. This inhibition was overcome by removing molybdate by centrifugal gel filtration, or by increasing the KCl concentration to 0.8 M. The inhibitory effects of molybdate on salt induced receptor disaggregation into activated subunits are no longer observed at pH > 7.4 or after chemical modification of sulfhydryl groups. Once androgen receptor has been disaggregated into its activated subunits the activated state is maintained even upon reassociation to 10–11 S aggregates in low salt. The relative concentrations of KCl and molybdate are critical; thus, 10 mM Na_2MoO_4/0.4 M KCl and 50 mM Na_2MoO_4/0.8–1.2 M KCl did not differentiate activated from non-activated androgen receptor based on their hydrodynamic properties. In the presence of 0.4 M KCl and 50 mM molybdate, however, the hydrodynamic properties of androgen receptor can be correlated with receptor activation.

INTRODUCTION

Although steroid hormone receptors have been extensively characterized (for review cf. 1), the presence of multiple forms of these receptors, with molecular weights ranging from 30,000 to 350,000 Daltons [2–4], sedimentation coefficients from 2.6 S to 12 S [5–7] and Stokes radii from 23 Å to 83 Å [2–6] has made it difficult to relate physiological function to any specific receptor state. Variations in the experimental conditions [7], proteolysis of receptor [2, 5], and changes in receptor properties after activation and transformation are thought to be the factors responsible for these varying steroid receptor forms [3, 6]. Thus, appropriate experimental conditions must first be established to allow the study of activation, nuclear translocation and binding to acceptor sites.

Activation and/or transformation of rat ventral prostate androgen receptor is defined as the ability of the hormone receptor complex to bind nuclei, DNA and other polyanions. Although studies on androgen receptor activation have suggested that receptor dissociates into subunits, activated and nonactivated androgen receptors cannot be distinguished through their hydrodynamic properties in conventional sucrose density gradients or gel filtrations. This is due to dissociation of nonactivated androgen receptors in high salt and reaggregation of activated receptors in low ionic strength buffers. Thus, a correlation between androgen receptor hydrodynamic properties and activation or transformation has not yet been established.

Using sodium molybdate as stabilizing agent, and leupeptin and phenylmethyl sulfonylfluoride as proteolysis inhibitors, we have investigated the hydrodynamic parameters of rat ventral prostatic androgen receptor and the relation of these parameters to activation (nuclear binding). This has allowed us to attribute specific sedimentation coefficients and Stokes radii to the activated and non-activated species of the receptor.

Thus, we have determined the experimental conditions which will allow resolution of activated and nonactivated androgen receptor complexes by high salt sucrose density gradients or gel filtration.

EXPERIMENTAL

Isotopes and chemicals: [1,2-^{3}H]5α-dihydrotestosterone [^{3}H]DHT; 51.6 Ci/mmol); (1,2,4,5,6,7-^{3}H]-

DHT, (143 Ci/mmol), and [^{14}C]glycine were obtained from New England Nuclear Corp., (Boston, MA). Nonradioactive DHT was obtained from Steraloids (Wilton, NH). All other reagents used were of analytical grade and obtained from commerical sources. Bovine serum [^{14}C]albumin, human [^{14}C]γ-globulin and [^{14}C]ovalbumin were prepared according to the method of Jentoft and Dearborn[8].

Animals

Male Sprague–Dawley rats (250–300 g b.wt) were purchased from Charles River Breeding Laboratories (Wilmington, MA) or from ESS Laboratories (Lynnfield, MA). All animals were castrated via the scrotal route 18–24 h before use. Animals were sacrificed and the ventral prostate removed and either used immediately or stored in liquid nitrogen for 1–2 weeks.

Buffers

Buffer TEG; [50 mM Tris–HCl, 1.5 mM EDTA, 10% (v/v) glycerol 0.02% (w/v) NaN_3; pH 7.2 at 0–2°C]. Sodium molybdate was added to the buffer to give a final concentration of 10 or 50 mM as specified for each experiment. Dithiothreitol (DTT, 1 mM) or monothioglycerol (MTG, 10 mM) was added to the buffer just before use. All homogenization buffers contained 0.5 mM phenylmethyl sulfonylfluoride (PMSF) and 1 mM leupeptin to inhibit receptor proteolysis.

Tissue homogenization and preparation of subcellular fractions

Unless otherwise stated all manipulations were carried out at 0–4°C. Frozen prostatic tissue samples were pulverized using a Thermovac tissue pulverizer. Fresh tissue or frozen powder was homogenized in 3–4 vol of buffer using a Duall glass–glass homogenizer with a motor driven pestle. The homogenate was centrifuged at 800 *g* for 10 min. The supernatant was recentrifuged at 100,000 *g* for 30 min to yield the high speed supernatant (cytosolic fraction) and microsomal pellet. The 800 *g* pellet was washed three times by resuspension in buffer followed by centrifugation.

Binding of [^{3}H]DHT to cytosolic androgen receptor

Nine volumes of cytosol were mixed with 1 vol of $1–2 \times 10^{-7}$ M [^{3}H]DHT prepared in the same buffer and incubated at 0°C for 2–3 h. Parallel incubation was made with [^{3}H]DHT and 200-fold molar excess of unlabeled DHT to determine nonspecific binding. Binding of [^{3}H]DHT to cytosolic androgen receptor was measured by HAP adsorption technique [9, 10]; specific binding is the difference between [^{3}H]DHT bound without and with a 200-fold molar excess of unlabeled DHT.

Nuclear binding assay of unactivated and activated androgen receptor

Activation of cytoplasmic [^{3}H]DHT receptor complexes was evaluated by measuring their ability to bind to nuclei during a 1 h incubation at 0°C.

Nuclei were suspended in buffer TEG containing 10 mM molybdate and 1×10^{-8} M DHT. As already observed with the estrogen receptor [11], inclusion of molybdate prevents secondary activation of non-activated androgen receptor which takes place at 0°C during the 1 h incubation with nuclei. Unlabeled DHT was added to assure that any cytosol receptors which might contaminate the nuclear preparation do not bind to [^{3}H]DHT upon addition of [^{3}H]DHT-labeled cytosol. To determine receptor activation cytosol, which had been incubated with [^{3}H]DHT in the presence or absence of unlabeled DHT, was treated with charcoal and was added to an equal volume of nuclear suspension (150–300 μg DNA). To determine activation of 0.4 M KCl treated cytosol, the salt concentration was first reduced by dilution to 0.1 M, a value which does not interfere with nuclear binding. At the end of the incubation, nuclei were sedimented by centrifugation at 1500 *g* for 5 min at 0°C and washed 3 times by resuspension and centrifugation. The washed nuclear pellet was extracted with 2 ml of abs. ethanol at 37°C for 1 h, and the total extract was transferred to scintillation vials containing 10 ml of Betafluor (National Diagnostics, NJ). [^{3}H]DHT associated with the nuclear fraction represents binding of activated androgen receptor complexes. Nonspecific binding of [^{3}H]DHT in cytosolic and nuclear fractions was $< 10\%$ of total bound [^{3}H]DHT. The concentration of labeled receptors in the cytosol was measured by HAP assay; nuclear binding is expressed as percentage of cytosolic R [^{3}H]DHT which was added to nuclei.

Miscellaneous

Sucrose density gradient analysis and gel filtration on Sephacryl-S200 were performed as described previously [10]. Estimation of S values, molecular weights and Stokes radii was made according to Martin and Ames[12], Siegel and Monty[13], and Sherman[14], respectively.

Protein was measured by the method of Lowry *et al.*[15], DNA by that of Burton[16].

RESULTS AND DISCUSSION

Cytosolic androgen–receptor complexes formed at 0°C (non-activated and non-transformed state) have sedimentation coefficients ranging from 8 S to 12 S on low salt SDG [7, 17]; this variation is related to differences in experimental conditions, such as buffer composition, pH, or length and temperature of incubation of cytosol with androgen [7]. Sedimentation on high salt SDG (0.4 M KCl) causes disaggregation into 4.4–4.6 S subunits [7, 17]; smaller entities

(3–3.6 S) are also observed, but have been shown to represent proteolytic fragments of receptor [5]. The inclusion of sodium molybdate in all buffers was shown to prevent loss of steroid binding ability of most steroid receptors [18–22]. This has been attributed to inhibition of receptor degradation, although a direct effect of molybdate on proteolysis of receptors has not been demonstrated.

While the effect of molybdate on androgen binding to the receptor has been studied extensively [21–22], very little is known about the effects of this metal ion on the hydrodynamic properties of this receptor. We have found (data not shown) that the presence of sodium molybdate (10 or 50 mM) in homogenization, incubation, and sucrose density gradient buffers does not significantly alter the sedimentation properties of androgen–receptor complexes in low salt SDG ($10\,S \pm 1$; $n = 6$), an observation which agrees with previous reports [9, 21]. In contrast, molybdate greatly affects the sedimentation rate of androgen receptor on high salt (0.4 M KCl) SDG. Androgen–receptor complexes prepared without molybdate sediment at 4.5 S (Fig. 1A). While inclusion of 10 mM Na_2MoO_4 in the 0.4 M KCl/SDG (Fig. 1B) had no effect on the sedimentation rate (4.4 S), with 50 mM molybdate two distinct peaks (4.5 S and 7 S) of specifically bound [^{3}H]DHT were observed (Fig. 1C). It is possible that 50 mM sodium molybdate partially prevents KCl (0.4 M) from disaggregating the 10 S R [^{3}H]DHT into 4.5 S subunits.

When molybdate was present only in the homogenization and incubation buffers but not in the KCl–sucrose gradient, a 5.6 S peak was observed (Figs 1D and 1E). This receptor form may correspond to the 4.5 S entity of Fig. 1A, but, due to treatment with molybdate prior to centrifugation, it shows a slight increase in buoyant density. A similar effect of molybdate on the sedimentation rates of glucocorticoid receptor in high salt SDG has recently been reported [23]; in that study the S value increased from 3.8 S to 5.2 S. The sedimentation rates of proteins have been shown to be affected by metal ions, due to solvation changes [24].

Figures 1 F–I illustrate the effect of sodium molybdate in both the homogenization and gradient buffers. When 10 mM sodium molybdate was added to the gradient (Figs 1F and 1G) only the 5.6 S entity was observed; since this value is identical to that obtained with molybdate in the incubation buffer but not in the sucrose gradients (Figs 1D and 1E) it appears that the exposure of cytosol to molybdate (10 or 50 mM) prior to receptor centrifugation in high salt, is responsible for the appearance of the 5.6 S entity. When the concentration of molybdate in the high salt SDG was increased to 50 mM, only the 7–8 S receptor form was observed (Figs 1H and 1I). By comparing the sedimentation pattern on panel 1C with that of panels 1H and 1I it becomes apparent that the presence of 50 mM molybdate in the gradients counteracts the salt-induced disaggregation of

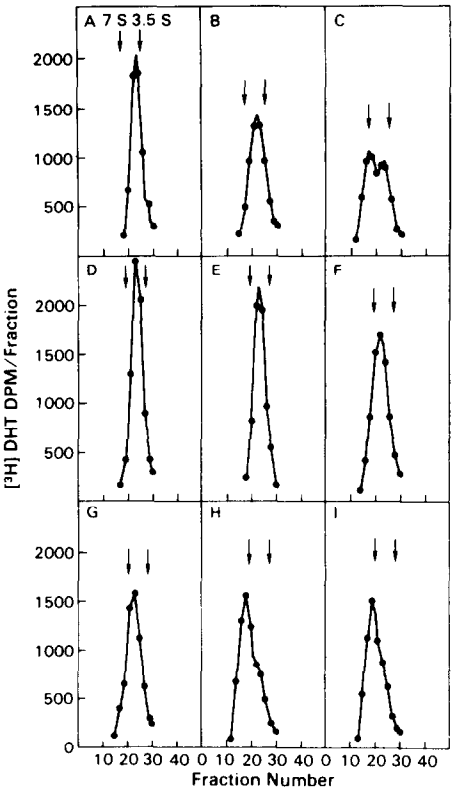

Fig. 1. Effect of molybdate on the sedimentation characteristics of androgen receptor complexes. Cytosol (8–10 mg protein/ml) is prepared in buffer TEG (panel A, B, C), TEG + 10 mM molybdate (panel D, F, H), or TEG + 50 mM Na_2MoO_4 (panel E, G, I). After incubation with [^{3}H]DHT at 0°C for 2 h, samples were treated with DCC and 0.1 ml aliquots were layered on SDG containing 0.4 M KCl without molybdate (A, D, E), with 10 mM molybdate (B, F, G) or with 50 mM molybdate (C, H, I). Parallel incubations with [^{3}H]DHT and an excess of unlabeled DHT were performed to determine nonspecific binding. All data represent specific binding. [^{14}C]γ-Globulin (7 S) and [^{14}C]ovalbumin (3.5 S) were used as internal sedimentation markers.

androgen receptor. Opposing effects of salt and molybdate on receptor dissociation have been reported previously for the estrogen, progesterone, and glucocorticoid receptors [25–27]. A significant difference between those receptors and the prostatic androgen receptor, however, is that with the latter higher concentrations of molybdate (50 mM) are required to prevent the salt induced disaggregation.

The ability of sodium molybdate to prevent loss of androgen receptor from kidney cytosol preparations has been shown to have a pH optimum between 6.6–7.1 [22]. We have recently found (data not shown) that with 50 mM molybdate in all buffers the receptor remains in the 7–8 S aggregated form only at pH 6.8–7.4; lower pH causes significant ligand dissociation while at pH 7.8–8.5 receptor disaggregation into 5.5 S entities takes place. Thus, the ability of molybdate to inhibit salt induced 8 S–5 S disaggregation is optimal at neutral pH.

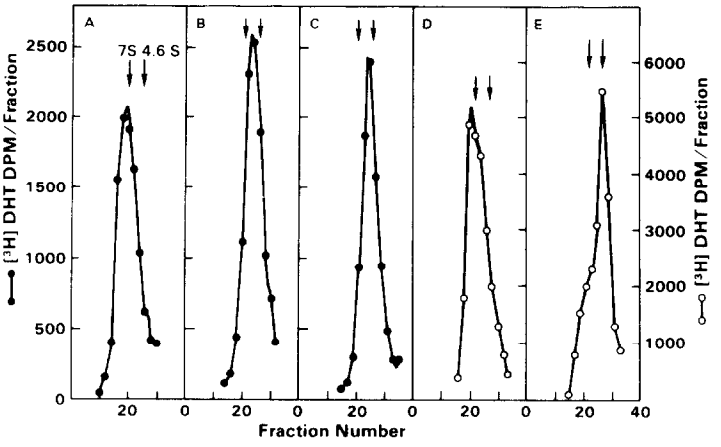

Fig. 2. Reversibility of molybdate action by increased ionic strength or upon its removal by gel filtration. Cytosol (8–11 mg protein/ml) prepared in buffer TEG containing 50 mM molybdate was incubated with [³H]DHT at 0°C for 2 h; some samples were treated with DCC and layered (0.1 ml) on SDG containing 50 mM molybdate with 0.4 M KCl (A), 0.8 M KCl (B) or 1.2 M KCl (C); others were subjected to centrifugal gel filtration on Sephadex G-25 to remove molybdate. Upon reconstitution with 1/5 volume of 2 M KCl (E), or 2 M KCl with 250 mM molybdate (D) the samples were allowed to stand at 0°C for 2 h and 0.3 ml of each sample was layered on SDG containing 0.4 M KCl and 50 mM molybdate. All data represent specific binding.

The action of molybdate in maintaining estrogen, progesterone, and glucocorticoid receptors in the aggregated state can be reversed by removing the metal ion [25–27]; in the presence of molybdate prolonged incubation in high ionic strength buffers allows estrogen receptor disaggregation into activated subunits [28]. Data on the reversibility of the molybdate effects on the androgen receptor are presented in Fig. 2. Cytosolic androgen receptor complexes were prepared at 0°C and analyzed on 0.4 M KCl/SDG containing 50 mM sodium molybdate; under these conditions (Figs 1I and 2A) R [³H]DHT sedimented in the 7–8S region; KCl concentrations of 0.8 M (Fig. 2B) or 1.2 M (Fig. 2C) resulted in the appearance of an entity with a sedimentation coefficient of 5.3–5.6. This presents further evidence that high ionic strength and molybdate have an opposing effect on the disaggregation of the 8–12 S androgen receptor complexes formed in hypotonic buffers; prolonged exposure to 0.8–1.2 M KCl during centrifugation completely reversed the effects of molybdate (Figs 2, B–C).

The effect of removing molybdate was examined by using centrifugal gel filtration [10, 28]. Cytosolic R [³H]DHT prepared in low salt buffer containing 50 mM molybdate was centrifuged through a Sephadex G-25 column to remove molybdate without reducing the protein concentration [10, 28]. The samples were collected during gel centrifugation into test tubes containing 2 M KCl buffer with or without 250 mM sodium molybdate to give final concentrations of 0.4 M KCl and 0/50 mM molybdate. Because of the brief centrifugation time (2 min) rapid salt reconstitution (with or without molybdate) was achieved. After 2 h at 0°C each sample was centrifuged through a 0.4 M KCl/SDG containing 50 mM molybdate. Figure 2D shows that immediate reconstitution with molybdate after centrifugal gel filtration prevented KCl induced disaggregation of R [³H]DHT (7–8 S), while lack of molybdate allowed dissociation of the majority of the receptor into 4.5 S entities (Fig. 2E); it is likely that during rapid gel filtration the unbound molybdate was removed from the cytosol while protein bound molybdate was not dissociated. During the subsequent incubation with KCl molybdate dissociated from most of the receptors and disaggregation into (4.5 S) subunits took place; the faster sedimenting entity (cf. 7–8 S shoulder in Fig. 2E) represents receptor aggregates from which molybdate had not dissociated.

Effect of SH-directed reagents on the sedimentation of androgen receptor complexes in the presence of molybdate

Recent studies on molybdate stabilization of estrogen receptor of human breast cancer [29] suggest that the integrity of −SH groups is essential for the maintenance of the 8–9 S state. The inhibiting effect of molybdate on glucocorticoid receptor activation was also shown to require the integrity of −SH groups [30]. We have, therefore, investigated whether −SH modifying reagents would interfere with the ability of molybdate to maintain the androgen receptor in the 7–8 S state.

Prostatic cytosol was incubated for 2 h at 0°C with [³H]DHT in the presence of 50 mM molybdate. Sedimentation analysis (Fig. 3) was performed in sucrose density gradients prepared with 0.4 M KCl and 50 mM molybdate, with or without 5 mM nitrocyanobenzoic acid (NTCB), dithionitrobenzoic acid (DTNB), α-iodoacetamide (IA) or sodium tetrathionate (NaTT). While in the absence of these

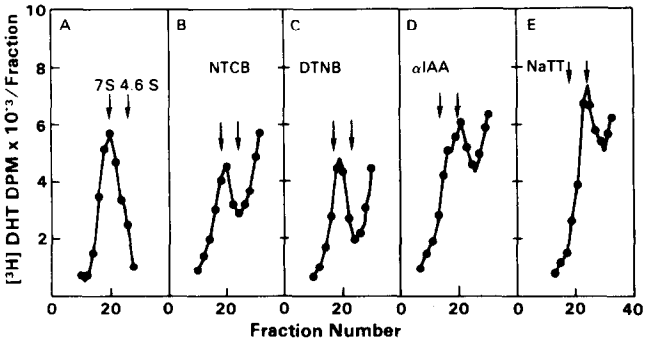

Fig. 3. Effect of −SH directed reagents on the ability of molybdate to prevent KCl-induced disaggregation of androgen–receptor complexes. Cytosol (8–11 mg protein/ml) was prepared in buffer TEG with 50 mM molybdate but without DTT or MTG. After incubation with [^{3}H]DHT for 2 h at 0°C samples were treated with DCC and 0.2 ml aliquots were layered on SDG gradients containing 0.4 M KCl and 50 mM molybdate without (A), or with 5 mM NTCB (B), 5 mM DTNB (C), 5 mMαIA (D), 5 mM NaTT (E). All data represent specific binding.

reagents (Fig. 3A) the androgen receptor sedimented at 7–8 S their inclusion in the sucrose gradient eliminated the ability of molybdate to prevent salt-induced dissociation of the androgen receptor into subunits. In the presence of NTCB or DTNB the receptor sedimented at 6 S (Figs 4B and 4C) and with the more reactive [31] reagents α-IA (Fig. 4D) or NaTT (Fig. 4C) complete dissociation into 4.5 S subunits was observed. This suggests that molybdate interacts with −SH groups to prevent salt-induced disaggregation of androgen receptors; however, unequivocal evidence for this assumption must await analysis with purified androgen receptor.

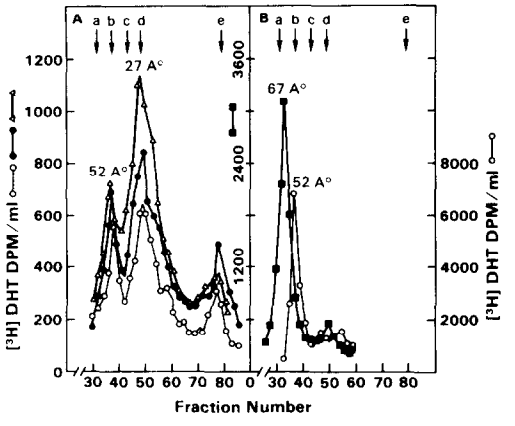

Fig. 4. Gel filtration of androgen receptor on Sephacryl-S200. Cytosol prepared with or without molybdate was labeled with [^{3}H]DHT for 2 h at 0°C and treated with DCC. Aliquots (1–2 ml) were mixed with 0.2 ml of buffer containing blue dextran (a), human [^{14}C]γ-globulin (b), bovine [^{14}C] serum albumin (c), [^{14}C]ovalbumin (d), and [^{14}C]glycine (e). The sample was then applied to a Sephacryl S-200 column (85 × 2.6 cm) preequilibrated and eluted with TEG buffer containing 0.4 M KCl with or without molybdate as specified below; flow rate was 0.2 ml/min. Fractions (4.6–4.8 ml) were collected and 1 ml of each fraction was taken for radioactivity counting. Panel A: Cytosol prepared and eluted with buffer without MoO_4 (○---○); cytosol in 50 mM molybdate, elution buffer without molybdate (●---●); cytosol without molybdate, elution buffer with molybdate (△---△). Panel B: cytosol prepared in 50 mM molybdate and eluted with buffer containing 50 mM molybdate; with 0.4 M KCl (■---■) or 0.8 M KCl (○---○).

Gel filtration analysis of androgen receptor in the presence of molybdate and KCl

In the absence of molybdate three forms of androgen receptor have been found in high ionic strength buffers, with Stokes radii of 54, 37 and 23 Å [4]. The two smaller moieties were shown to be proteolytic fragments of the 54 Å receptor. We had previously suggested [10] that molybdate stabilizes the androgen receptor in a large molecular weight form having a Stokes radius of 65–68 Å; this entity did not dissociate into subunits when exposed to KCl and molybdate during gel chromatography.

A more detailed analysis of the molecular entities observed when R[^{3}H]DHT is prepared with or without 50 mM molybdate and chromatographed on Sephacryl S-200 using 0.4 M KCl buffer in the presence or absence of 50 mM molybdate is shown in Fig. 4. The omission of sodium molybdate from all buffers (open circles), or only from the homogenization and incubation buffers (open triangles), or only from the elution buffer (solid circles), yields two receptor forms with Stokes radii of 52 Å and 27 Å, respectively (Panel A). This suggests that the absence of molybdate during receptor preparation and/or receptor chromatography results in formation of proteolytic fragments (27 Å); the 52 Å entity probably represents the product of salt-induced disaggregation of receptor during chromatography. These two receptor forms detected on gel chromatography (52 Å and 27 Å) cannot be separated on sucrose density gradients (Fig. 1). With the glucocorticoid receptor 51 Å and 32 Å entities were also detected by gel-filtration analysis, whereas only a single 4.4 S peak was detected on sucrose gradients [2].

When androgen receptor was prepared in buffer containing molybdate and chromatographed in the presence of high salt (0.4 M KCl) as well as 10 mM (data not shown) or 50 mM molybdate (Fig. 4B, solid squares) only the aggregated form, with an estimated

Stokes radius of 67 Å ± 1.4 ($n = 7$), was observed. Under identical conditions (Figs 1 and 2) this receptor sedimented at 7–8 S. Thus, sodium molybdate prevented the salt induced disaggregation of the 67 Å receptor into the 52 Å entity. Increasing the KCl concentration in the elution buffer to 0.8 M (open circles) yielded a 52 Å entity; this ionic strength also induced disaggregation of the 7–8 S molybdate-stabilized receptor into a 5.5 S entity (Fig. 2B). Furthermore, the presence of molybdate during receptor preparation and elution from the gel prevented formation of the 27 Å fragment, presumably through inhibition of proteolysis. These chromatographic patterns are similar to those obtained for glucocortoid receptor [2, 3].

Finally, R [^{3}H]DHT complexes formed at 0°C in buffer containing 10 mM sodium molybdate were analyzed by isoelectric focusing according to the method of Wrange and Yu[32]. The specifically bound [^{3}H]DHT migrated to a position corresponding to an isoelectric point of 6.5 (Fig. 5). No other peaks were observed, again suggesting that molybdate prevented receptor proteolysis [32]. Whether the receptor remained in the aggregated state or dissociated into subunits during electrofocusing remains to be determined.

Correlation between activation and sedimentation rates

Activation, an increase in affinity of the receptor for nuclei, chromatin, or polyanions, is commonly induced by exposing androgen receptor complexes formed at 0°C in hypotonic buffer to temperatures of 15–25°C or to salt molarities of 0.3–0.5 M. Activated and non-activated receptors, however, cannot be distinguished on the basis of their sedimentation rates in conventional sucrose density gradients. For instance, non-activated (21% nuclear binding) and heat-activated (43% nuclear binding) R[^{3}H]DHT

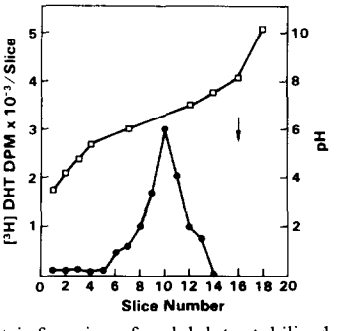

Fig. 5. Isoelectric focusing of molybdate stabilized androgen receptor complexes. Cytosolic androgen receptor prepared in TEG with 10 mM molybdate and labeled with [^{3}H]DHT was subjected to isoelectrofocusing on thin polyacrylamide gel plates (pH 3–10) at 0–2°C according to Wrange and Yu [32]. The gel was sliced into 4 mm sections, extracted, and counted. pH was measured with a surface electrode at 2°C. Markers were used in parallel lanes to estimate the pI of the androgen receptor. All data represent specific binding. Arrow represents point of application of the sample.

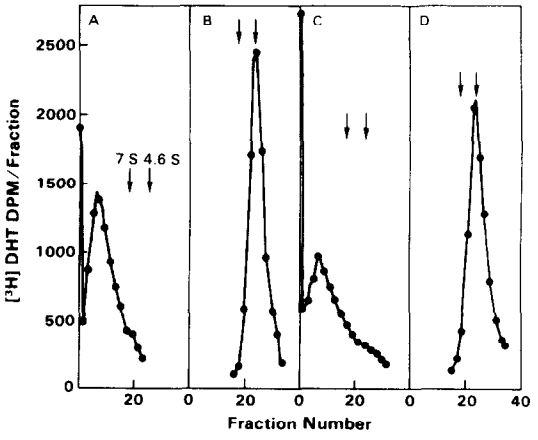

Fig. 6. Sedimentation of unactivated and activated androgen receptor complexes on low and high salt gradients without molybdate. Cytosol (8–10 mg protein/ml) was prepared without molybdate and incubated with [^{3}H]DHT for 90 min at 0°C. Aliquots were either kept at 0°C (A, B) or heated for 30 min at 18°C for activation (C, D). After DCC treatment aliquots were centrifuged on SDG without (A, C) or with (B, D) 0.4 M KCl. All data represent specific binding.

sediments at 10–11 S in low, and 4.6 S in high salt SDG (Fig. 6). It is generally assumed that 10–11 S is the sedimentation rate of non-activated receptor on low salt SDG; this, however, may be erroneous since during centrifugation the androgen receptor might have undergone conformational changes resulting in activation. To resolve this point non-activated R [^{3}H]DHT was kept at 0°C for 24 h in 16% sucrose to mimic the conditions to which receptor was exposed during the centrifugation analysis which yielded the 11 S entity (Fig. 6). That activation took place is suggested by the observation that 54% of this R [^{3}H]DHT was able to bind to nuclei (Table 1). It is most likely, therefore, that also the 11 S entity obtained upon centrifugation of non-activated receptor (Fig. 6A) reflects conversion to activated receptor. Further verification was obtained by centrifugation of non-activated androgen receptors on low salt gradients (Fig. 7A). Receptors sedimenting in the 10–11 S region of the gradients were pooled and added to nuclei; 60% of these R [^{3}H]DHT complexes bound to nuclei, as compared to 13% for the molybdate stabilized 10 S complexes (Fig. 7B).

The molecular mechanism of receptor activation involves conformational changes of the nuclear binding domain resulting in the exposure of positively charged amino acid residues [33, 34]; this nucleotropic domain interacts with nuclear acceptors. It is likely that a dynamic equilibrium exists between aggregated and disaggregated receptor. We suggest that whenever non-activated 10–11 S receptor dissociates during prolonged incubation at 0°C in hypotonic buffers, the subunits undergo those conformational changes required to expose the nuclear binding domain; reassociation of these activated subunits yields 10–11 S receptor aggregates with in-

Table 1. Effects of various treatments on the activation of androgen receptors prepared with and without molybdate

Treatment	% Of cytosolic androgen receptors bound to nuclei and their respective S values			
	$-MoO_4^{2-}$		$+50$ mM MoO_4^{2-}	
	Nuclear binding	(S Values)	Nuclear binding	(S Values)
Cytosol +[^{3}H]DHT, 0°C, 2 h,	21	(7.4 S + 4.6 S)	3	7.4 S
Cytosol +[^{3}H]DHT, 0°C, 1 h, 18°C, 1 h	43	4.6 S	3	7.2 S
Cytosol +[^{3}H]DHT, 0°C, 1 h, 0.4 M KCl, 1 h	38	4.6 S	2	7.4 S
Cytosol +[^{3}H]DHT, 0°C, 1 h, 0.4 M KCl 18°C, 1 h	39	4.6 S	8	7.4 S
Cytosol +[^{3}H]DHT, 0°C, 1 h, 1:1 dil. with buffer +32% sucrose, 24 h	54	ND	6	ND
Cytosol +[^{3}H]DHT, 0°C, 1 h, 1:1 dil. with buffer +0.8 M KCl, 32% sucrose 24 h	41	ND	6	ND

Cytosols (8–12 mg protein/ml) prepared in buffer TEG without or with 50 mM molybdate were incubated with [^{3}H]DHT at 0°C for 1 h. At this point aliquots were removed and treated as described above. At the end of each treatment aliquots of each incubation were added to nuclei to measure activation and parallel aliquots were assayed for bound radioactivity with HAP. Similar aliquots were analyzed on sucrose density gradients containing 0.4 M KCl and 50 mM molybdate. Specifically bound [^{3}H]DHT in the nuclei was calculated as % of total specifically bound [^{3}H]DHT added to nuclei.

creased affinity for nuclei. This could explain the observation that prolonged incubation at 0°C in hypotonic buffer (Table 1), or centrifugation on low salt gradients, increased nuclear binding of androgen receptors to 54%. Similar observations have been made for the estrogen receptor [35].

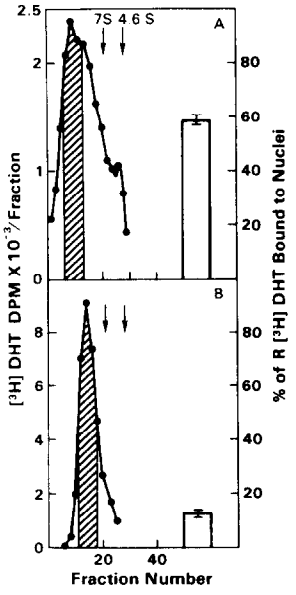

Fig. 7. Nuclear binding of androgen receptor complexes sedimenting at 10–11 S. Cytosols prepared in buffer without (A) or with molybdate (B) were labeled with [^{3}H]DHT at 0°C for 1 h. After DCC treatment aliquots (0.3 ml) were layered on low salt SDG prepared in the corresponding buffers and centrifuged at 53,000 rpm for 18 h at 2°C. Material from 3 gradients was collected into individual tubes containing 50 μl of buffer with 100 mM molybdate (to stop any activation beyond this point). Fifty μl samples were removed and radioactivity counted. Fractions 6–13 (panel A) and 11–18 (panel B) were pooled and 0.8 ml of each was added to 0.8 ml of nuclear suspension to assay for receptor activation. Androgen receptor content of the pooled fractions was assayed by HAP adsorption. All data represent specifically bound [^{3}H]DHT.

Table 1 shows that activation, regardless of the induction process, is completely inhibited by 50 mM sodium molybdate. Thus neither heat (18°C), prolonged incubation at 0°C in hypotonic buffers, nor prolonged incubation (24 h) at 0°C in 0.4 M KCl–16% sucrose resulted in receptor activation in the presence of sodium molybdate (50 mM); these non-activated R [^{3}H]DHT complexes remain in the aggregated 7–8 S state when sedimented on 0.4 M KCl–50 mM Na_2MoO_4 SDG. In contrast, on identical gradients heat-activated receptor (50% nuclear binding) sedimented at 4.6 S $\pm$ 0.2, ($n = 8$). Lower molybdate concentrations (10 mM) and prolonged incubation in 0.4 M KCl, however, did permit partial activation (data not shown). Thus, while heat activated and non-activated androgen receptors cannot be differentiated on conventional sucrose gradients (Fig. 6; 4.6 S on high salt SDG, 10–11 S on low salt SDG) these two receptor states can be distinguished on 0.4 M KCl–50 mM molybdate SDG.

CONCLUSIONS

"Activation" refers to changes in the properties of steroid receptors which are recognized by an increased affinity of the hormone receptor complex for nuclei, chromatin, and other negatively charged acceptor systems. In contrast to other steroid receptors a relationship between activation and specific changes in the hydrodynamic properties (i.e. transformation) of the androgen receptor could not be demonstrated, so far. As shown here, activated and non-activated androgen receptor cannot be distinguished by sedi-

mentation analysis on conventional low salt or high salt sucrose density gradients. Similarly, activated and non-activated receptors cannot be distinguished by conventional gel-filtration [4, 5]. However, under carefully selected conditions one can recognize those changes in the hydrodynamic characteristics of androgen receptors which are concomitant with its transition from the non-activated to the activated state. Thus, in the presence of 0.4 M KCl and 50 mM Na_2MoO_4 non-activated receptor has a Stokes radius of 67 Å, a mol. wt of 198,000 Daltons, and a sedimentation coefficient of 7–8 S; upon salt-activation the Stokes radius decreases to 52 Å, the mol. wt to 98,000 Daltons and the sedimentation coefficient to 4.6 S. Thus it is likely that the non-activated receptor is a dimer.

As previously observed with the estrogen receptor [11, 28], sodium molybdate also prevents activation of androgen receptors by maintaining the receptor in the aggregated 7–8 S state. The disaggregation into activated subunits induced by high ionic strength was also inhibited by sodium molybdate (Figs 1 and 2); this inhibition requires the integrity of the receptor's sulfhydryl groups. The effects of molybdate are reversible upon its removal or by increasing the KCl concentration to >0.8 M. The opposing effects of KCl and sodium molybdate on receptor activation and sedimentation strictly depend upon the concentrations of each of these two reagents. Finally, once receptor aggregates (10–11 S on low salt SDG, 7–8 S in high salt/molybdate SDG) have been dissociated into activated subunits (increased nuclear binding and 4–5 S sedimentation coefficient in high salt/molybdate SDG) the activated state is maintained even upon reaggregation to a 10–11 S form. Using the methodology described in this paper, it is now possible to distinguish activated and non-activated androgen receptor on the basis of their distinct hydrodynamic properties.

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