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CHARACTERIZATION OF NON-LIGANDED GLUCOCORTICOID RECEPTOR IN RAT LIVER CYTOSOL USING INDIRECT COMPETITIVE ENZYME–LINKED IMMUNOSORBENT ASSAY

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Summary—We have previously shown that the purified or unfractionated cytosolic, activated glucocorticoid receptor of rat liver consists of a polypeptide with a Stokes radius of ~6 nm, a sedimentation coefficient of 4 S and a molecular mass of ~90,000 Daltons. We have confirmed previous observations by other authors that if sodium molybdate is introduced into the cytosol preparation buffer the non-activated glucocorticoid receptor appears as an 8 nm, 9 S species with an apparent molecular mass of 330,000 Daltons.

In order to study the physicochemical parameters of the glucocorticoid receptor prior to ligand binding, we have used an enzyme-linked immunosorbent assay (ELISA) based on antibodies raised in rabbits against the purified activated glucocorticoid receptor. In isotonic buffer, the non-liganded glucocorticoid receptor was shown to have a Stokes radius of 6 nm in the absence and 8 nm in the presence of molybdate. Furthermore, experimental conditions known to result in activation of the glucocorticoid receptor complex (increased ionic strength, increased temperature) did not lead to activation of the 6 nm non-liganded glucocorticoid receptor as judged from the lack of binding of the treated, non-liganded receptor to DNA-cellulose.

The existence of both 6 and 8 nm forms of nonactivated, non-liganded glucocorticoid receptor *in vitro* suggests that dissociation of an 8 nm form to a 6 nm form, if it occurs *in vivo*, is probably not the only molecular event constituting the activation of the glucocorticoid receptor.

INTRODUCTION

The crucial role of steroid receptor proteins as cellular information transfer factors is now generally recognized. A precise knowledge of the structural and functional features of steroid receptors would be of significant help for a better understanding of the molecular mechanisms of steroid hormone action.

The pronounced instability of steroid receptors in non-liganded state [1–3] as well as their liability to become activated (i.e. to gain the capacity to bind to DNA) after ligand binding even at low temperatures [4, 5] have significantly hampered efforts to characterize non-activated receptors. This problem was partly solved by using sodium molybdate, which was shown to have remarkable effects on both functional and structural characteristics of all classes of steroid receptors [6–16]. Molybdate preserves the ligand binding capacity of non-liganded glucocorticoid receptor [8] and facilitates the reactivation of inactivated receptor to the steroid binding state by reducing agents like dithiothreitol [6, 7, 17, 18]. Simultaneously, it inhibits activation of the ligand–glucocorticoid receptor complex to the DNA-binding state [8].

Analysis of the glucocorticoid receptor in hypotonic buffer containing sodium molybdate virtually eliminated the multiplicity of receptor forms and/or fragments reported previously [19–23]. In the presence of sodium molybdate, glucocorticoid receptor appeared as a species with a Stokes radius of ~8 nm, a sedimentation coefficient of ~9 S and a calculated mol. wt of ~330,000 Daltons [24].

The characteristics of molybdate-stabilized receptors were constant and independent of sex, tissue (liver, mammary tumor, lymphocytes) and species (man, mouse, rat) [14, 15]. The oligomeric form of the receptor was observed in the absence of molybdate as well. As similar data were obtained for all classes of steroid receptors studied (glucocorticoid, estrogen, progestin) [14–16, 24] they were taken as an indication for the existence of a common structure of all steroid receptors which is stabilized in the presence of molybdate.

We have reported that the purified, activated glucocorticoid receptor from rat liver cytosol consists of a single ~90 K polypeptide with a sedimentation coefficient of 4 S and a Stokes radius of 6.0 nm [25].

The structural and functional relationship between the activated 6 nm glucocorticoid receptor and the non-activated molybdate stabilized 8 nm glu-

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Abbreviations: ELISA, enzyme-linked immunosorbent assay Triamcinolone acetonide, (9α-fluoro-11β,21-dihydroxy-16α,17α-[(1-methylethylidene)*bis*(oxy)]-1,4-pregnadiene-3,20-dione).

cocorticoid receptor has so far been unclear, although it has been suggested that the molybdate stabilized glucocorticoid receptor could be an oligomer, presumably a tetramer [26–28], of the activated 90 K glucocorticoid receptor and that the activation process might involve the dissociation of the multimer, thus resulting in formation of the 6 nm activated glucocorticoid receptor "monomer" [26–29].

The requirement for the formation of a hormone–receptor complex prior to activation of the receptor in crude preparations was demonstrated previously for the glucocorticoid receptor [4, 30, 31]. This report demonstrates the use of an indirect competitive enzyme-linked immunosorbent assay (ELISA) in order to characterize the non-liganded and non-activated glucocorticoid receptor. This approach has allowed us to investigate the physicochemical state of the non-activated glucocorticoid receptor without the use of molybdate. The ELISA method is based on anti-glucocorticoid receptor-antibodies raised in rabbits against purified rat liver cytosolic glucocorticoid receptor protein [32].

EXPERIMENTAL

Chemicals

[6,7-^{3}H]Triamcinolone acetonide (specific radioactivity 1.10–1.85 TBq/mmol) was purchased from New England Nuclear (Boston, MA); it was routinely diluted to a specific radioactivity of 300 kBq/mmol using radioinert triamcinolone acetonide. Agarose A-1.5 m (100–200 mesh) was purchased from Bio-Rad (Richmond, CA). Sodium molybdate was obtained from Merck (Darmstadt, F.R.G.). Horse spleen ferritin, rabbit immunoglobulin G, ovalbumin, whale skeletal muscle myoglobin and dithiothreitol were obtained from Sigma Chemical Company (St Louis, MO). Peroxidase was obtained from Dako Immunoglobulin Ltd (Copenhagen, Denmark). Sources of other materials have been given previously [25, 32, 33].

Preparation of glucocorticoid receptor

All animals were adrenalectomized 4–5 days prior to the experiment. Rat liver cytosol was prepared in EPG buffer [20 mM sodium phosphate, pH 7.4, 1 mM Na_2EDTA, 10% (w/v) glycerol] containing 2 mM dithiothreitol and 0.02% (w/v) sodium azide, as previously described [32, 24]. The cytosol was incubated for 60 min at 0°C with 100 nM [^{3}H]triamcinolone acetonide.

Gel filtration

Gel filtration was carried out on Agarose A-1.5 m columns (50 × 2.6 cm) in EPG buffer containing 0.15 or 0.3 M NaCl, 0.02% (w/v) Na-azide and, when indicated, 20 mM sodium-molybdate. The elution speed was 5 cm/h. The concentration of NaCl in the cytosol samples was adjusted to 0.15 or 0.3 M by the addition of 5 M NaCl 15 min prior to chromatography. In case the chromatography was carried out in the presence of 20 mM Na-molybdate, the samples were preincubated at this concentration of molybdate for 1 h. The columns were calibrated using the following proteins: horse spleen ferritin, [^{14}C]methylated ovalbumin and whale skeletal muscle myoglobin. The Stokes radii for these proteins were 6.15, 2.86 and 2.01 nm, respectively [35]. [^{14}C]Methylated rabbit immunoglobulin G was also used for calibration. The Stokes radius of the latter was 5.22 nm [36].

Glycerol gradient centrifugation

Linear glycerol gradients, 5–20% (w/v), were prepared in EPG buffer, pH 7.4, containing 0.15 or 0.3 M NaCl and 20 mM Na-molybdate when indicated. After removal of unbound hormone using a dextran-coated charcoal adsorption technique [33], 0.2 ml aliquots of cytosolic [^{3}H]triamcinolone acetonide–glucocorticoid receptor complex were layered on the gradients and centrifuged for 14–16 h at 0–4°C in an SW 60 Ti rotor at 140,000 *g*. Fractions, 200 μl, were collected from the bottom of the gradient using a Beckman fraction recovery system.

Sedimentation coefficients of glucocorticoid receptor were determined according to the method of Martin and Ames [37] using [^{14}C]methylated β-amylase (sweet potatoes) [16], ovalbumin and carbonic anhydrase for calibration. Sedimentation coefficients of these proteins were 9.4, 3.5 and 3.2 S, respectively [35]. [^{14}C]Methylated rabbit immunoglobulin G, sedimentation coefficient 7.1 S, was also used for calibration [36].

DNA-cellulose chromatography

DNA-cellulose was prepared as described by Alberts and Herrick[38]. Ten ml cytosol was labelled as outlined above. Activation was performed either by incubation with 0.3 M NaCl for 60 min at 0°C or by heat treatment at 25°C for 30 min. When activation was carried out using high salt concentration, the cytosol was diluted in EPG-buffer, pH 7.4, containing 2 mM dithiothreitol, following the activation step to a final concentration of 50 mM NaCl, prior to application on the DNA-cellulose column.

The activated, labelled cytosol was then treated with dextran-coated charcoal and applied on a 10 ml DNA-cellulose column. After washing with two column volumes of EPG buffer, elution was performed with a linear 0–0.5 M NaCl gradient in EPG buffer (2 × 70 ml).

In case of non-labelled cytosol all steps were performed in the same way as described above, except that the cytosol was not preincubated with steroid. The samples obtained after fractionation were immediately incubated with 100 nM [^{3}H]triamcinolone acetonide for 1 h and treated with dextran-coated charcoal. Radioactivity was determined in 100 μl aliquots.

Enzyme-linked immunosorbent assay (ELISA)

After chromatography aliquots were taken from each fraction and assayed for radioactivity and/or immunoactivity, using an indirect competitive ELISA. The method is based on specific antibodies against glucocorticoid receptor raised in rabbits as already described [32]. Samples of 0.2 ml were incubated at 4°C overnight with 0.05 ml of antiserum (G4, protein concentration 10 mg/ml) purified on protein A-Sepharose and diluted 1:100 using phosphated balanced saline solution. After incubation, the amount of antibody not bound to antigen was measured on micro-ELISA plates coated with 20 ng of purified GR [25] in each well. Either swine anti-rabbit antibody conjugated with horse radish peroxidase (Dako Immunoglobulin Ltd, Copenhagen, Denmark) or protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) conjugated with alkaline phosphatase (prepared as described in Ref. 39 and 40) were used for visualization of the anti-receptor antibodies. The horse radish peroxidase activity was measured using 0.055% (w/v) 1,2-phenylenediamine dihydrochloride (Fluka AG, Chemische Fabrik, CH 9470 Buchs, Switzerland) and 0.009% (w/v) H_2O_2 as substrate. Product of enzymatic reaction was detected spectrophotometrically at 492 nm [32]. Alkaline phosphate activity was measured using 1 mg/ml *p*-nitrophenylphosphate (NPP) (Sigma Chemical Company, St Louis, MO) as a substrate [39, 40]. Product of enzymatic reaction was detected spectrophotometrically at 405 nm [40]. Both assays gave identical results.

RESULTS

Physicochemical measurements of liganded glucocorticoid receptor in the presence or absence of Na-molybdate

In order to compare the physicochemical characteristics of the cytosolic glucocorticoid receptor in the presence or absence of Na-molybdate we performed gel filtration analysis to determine the Stokes radius of the ligand-receptor complex and ultracentrifugal analysis to measure its sedimentation coefficient.

Chromatographic analysis performed in isotonic concentration buffer (0.15 M NaCl) in the absence of molybdate revealed the existence of a single peak of radioactivity and immunoactivity (the latter seen as a dip in absorbance at 405 nm—Fig. 1A) with a Stokes radius of 5.7 ± 0.2 nm ($n = 5$) corresponding to the "6.0 nm glucocorticoid receptor complex" previously described [29, 36, 43]. The sedimentation coefficient recorded under corresponding conditions was 4.2 ± 0.4 S ($n = 4$) (Fig. 1A—insert). The apparent molecular weight of liganded glucocorticoid receptor in the absence of molybdate was 101,000 Daltons, when calculated as previously described [19].

If 20 mM Na-molybdate was introduced into the preparation buffer described above and used throughout the course of chromatographic and ultracentrifugal analyses, the Stokes radius of the cytosolic receptor was 8.1 ± 0.2 nm ($n = 3$) [Fig. 1B] and the sedimentation coefficient 9.4 ± 0.9 S ($n = 4$) [Fig. 1B—insert]. The calculated molecular weight of

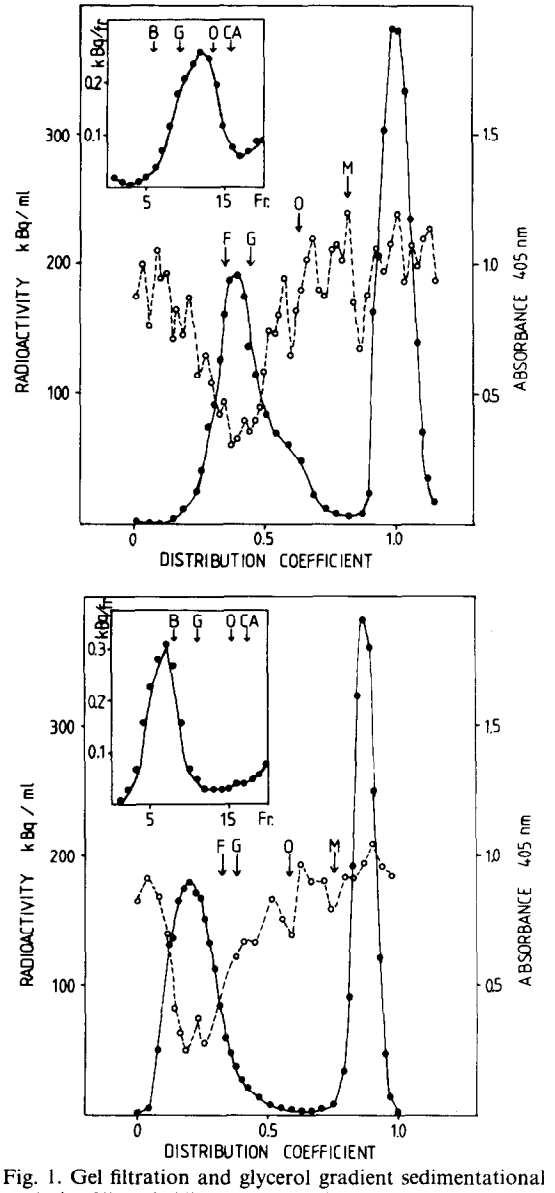

Fig. 1. Gel filtration and glycerol gradient sedimentational analysis of liganded liver cytosolic glucocorticoid receptor in the presence or absence of Na-molybdate. Cytosol was incubated with [^{3}H]triamcinolone acetonide as described in the Experimental section, the concentration of NaCl was adjusted to 0.15 M (A and B) and the concentration of Na-molybdate to 20 mM (B only). The samples were applied to Agarose A-1.5 m (see the Experimental section) and eluted with EPG buffer, pH 7.4, containing 2 mM dithiothreitol, 0.02% (w/v) Na-azide, 0.15 M NaCl (A and B) and 20 mM Na-molybdate (B). The chromatographic fractions were analyzed for content of radioactivity (filled symbols) and immunoactivity (open symbols). The trough seen in absorbance at 405 nm denotes presence of the receptor. Before application to the glycerol gradients (A and B inserts), samples were treated with dextran-coated charcoal. For details, see the Experimental section. Markers: F—ferritin, B—β-amylase, G—immunoglobulin G, O—ovalbumin, CA—carbonic anhydrase and M—myoglobin.

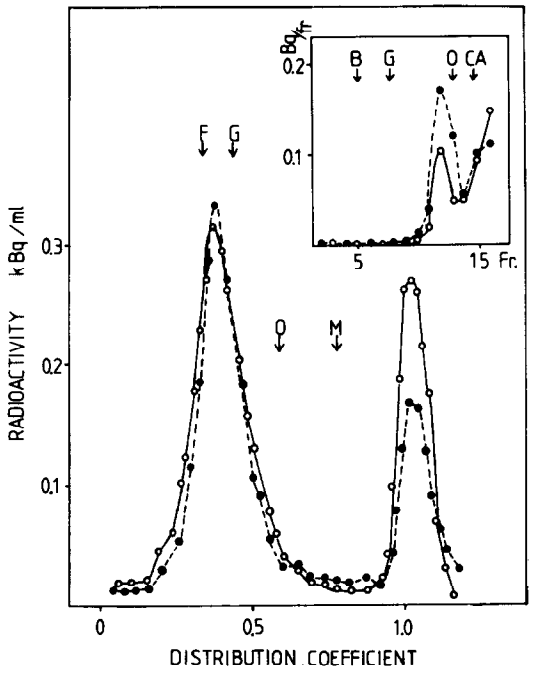

Fig. 2. Gel filtration and glycerol gradient sedimentational analysis of highly purified liver cytosolic glucocorticoid receptor in the presence or absence of sodium-molybdate. Highly purified activated [^{3}H]triamcinolone acetonide labeled glucocorticoid receptor (85% purity), containing 0.01% (w/v) insulin as a carrier, was incubated with (filled symbols) or without (open symbols) 20 mM Na-molybdate for 45 min at 0°C. Glucocorticoid receptor, 2 μg (protein concentration estimated as already described in ref. 25) was analyzed on Agarose A-1.5 m and 250 ng glucocorticoid receptor on a 5–20% (w/v) linear glycerol gradient (insert) in EPG pH 7.4 buffer containing 0.15 M NaCl in the presence or absence of Na-molybdate. The fractions were analyzed for content of radioactivity. For details, see the Experimental section. Markers: see Fig. 1.

the molybdate-treated ligand–receptor complex was approx 322,000 Daltons.

The physicochemical parameters recorded for both forms of glucocorticoid receptor were the same in the presence of either 0.15 or 0.3 M NaCl.

Chromatographic and sedimentational analyses of highly (up to 85% pure) purified activated rat liver cytosolic glucocorticoid receptor [25] under the same conditions as described above, both in the presence or absence of 20 nM Na-molybdate, showed the presence of one receptor form only. The form obtained in the absence of molybdate had a Stokes radius of 5.8 nm ($n = 2$) [Fig. 2], a sedimentation coefficient of 4.2 S ($n = 2$) [Fig. 2-insert] and a calculated mol. wt of 99,600 Daltons [19]. The form obtained in the presence of molybdate had a Stokes radius of 5.6 nm ($n = 2$), a sedimentation coefficient of 4.2 S ($n = 2$) and a calculated mol. wt of 96,500 Daltons [19].

Physicochemical measurements of non-liganded GR in the presence or absence of Na-molybdate

The availability of specific antibodies raised against purified glucocorticoid receptor [32] enabled us to study the behaviour of non-liganded receptor in the presence or absence of Na-molybdate.

The non-liganded glucocorticoid receptor in unfractionated cytosol was analyzed on Agarose A-1.5 m and followed by immunoactivity determined in each fraction using the ELISA technique described above. In the absence of Na-molybdate a single immunoactivity peak was observed corresponding to a Stokes radius of 5.7 nm [duplicate determinations] (Fig. 3A).

Addition of Na-molybdate throughout the course of the chromatographic analysis of non-liganded cytosolic receptor resulted in the appearance of a single peak of immunoactivity with a Stokes radius of 8.0 nm [single determination] (Fig. 3B).

Thus, both for liganded and non-liganded glucocorticoid receptor under our condition, the presence of the 6.0 or 8.0 nm forms of glucocorticoid receptor was dependent on whether Na-molybdate was absent or present, respectively.

DNA-cellulose binding of non-liganded and liganded glucocorticoid receptor

It has previously been reported that non-liganded calf uterus estrogen receptor may be activated *in vitro* to the DNA-binding state following ammonium sulfate precipitation [42]. Activation of non-liganded chick oviduct progesterone receptor was observed after ammonium sulfate precipitation [40], ATP treatment [43, 44] and treatment with high ionic strength or heparin [45]. In order to exclude the possibility that the non-liganded glucocorticoid receptor might have undergone activation under our experimental conditions, we performed DNA-cellulose chromatography of non-liganded receptor upon salt [0.3 M NaCl] (Fig. 4) or thermal (25°C) activation (not shown). Neither of these treatments resulted in any retention of glucocorticoid receptor on DNA-cellulose, as judged from the immunoactivity profile of the chromatogram (Fig. 4) or from the radioactivity profile obtained after immediate incubation of each fraction with the labelled steroid (not shown). Stokes radius of glucocorticoid receptor found in the DNA-cellulose flow through and labelled following this chromatography was 5.9 nm (measured in the absence of molybdate and in the presence of 0.15 M NaCl, not shown). Therefore, neither treatment with high salt concentration nor with high temperature led to activation, i.e. DNA-binding of non-liganded glucocorticoid receptor. The control experiments (Fig. 4) demonstrated that the same treatments of the [^{3}H]triamcinolone acetonide–glucocorticoid receptor complex led to receptor activation, as judged from the retained radioactivity/immunoactivity peak on DNA-cellulose, eluted at 0.17 M NaCl as previously reported [34].

If activation was attempted in the presence of Na-molybdate, neither non-liganded nor liganded glucocorticoid receptor bound to DNA-cellulose (not shown).

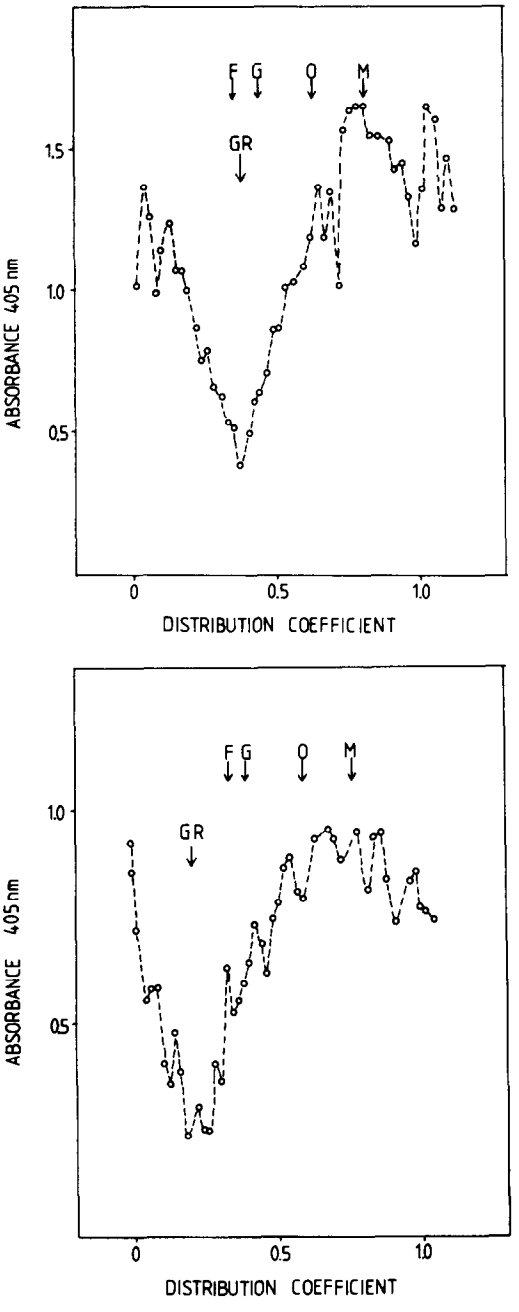

Fig. 3. Gel filtration of non-liganded cytosolic glucocorticoid receptor in the presence or absence of Na-molybdate. Rat liver cytosol was adjusted to 0.15 M with respect to NaCl (A and B) and to 20 mM with respect to Na-molybdate (B only). The samples were applied to Agarose A-1.5 m and eluted with EPG buffer, pH 7.4, containing 2 mM dithiothreitol, 0.02% (w/v) Na-azide, 0.15 M NaCl (A and B) and 20 mM Na-molybdate (B only). The chromatographic fractions were analyzed for immunoactivity by ELISA (open symbols). For further details, see the Experimental section.

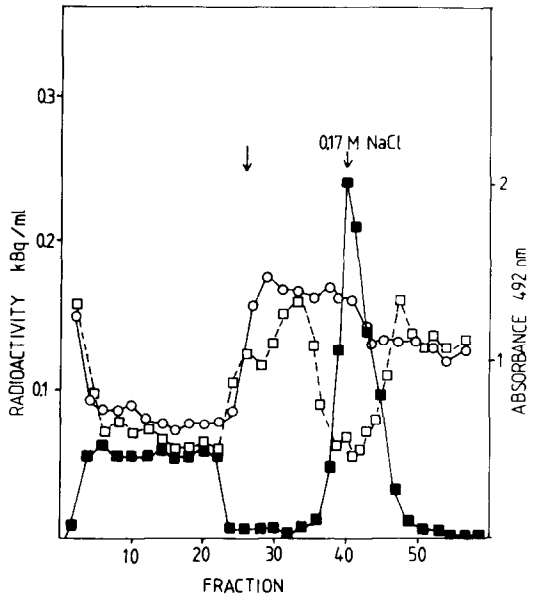

Fig. 4. DNA-cellulose chromatography of non-liganded glucocorticoid receptor. Cytosol was incubated with 0.3 M NaCl for 60 min and an aliquot was diluted to 50 mM NaCl using EPG pH 7.4 buffer, treated with dextran-coated charcoal and applied to DNA-cellulose equilibrated in EPG, pH 7.4, containing 2 mM DTT. Elution was performed with a linear 0–0.5 M NaCl gradient (for details, see the Experimental section). After fractionation, aliquots were assayed for content of immunoactivity (open circles). In the control sample, cytosol was first labeled with [^{3}H]triamcinolone acetonide for 60 min at 0°C and activated by 0.3 M NaCl whereupon the same steps were carried out as described above. After fractionation, aliquots were assayed for content of both radioactivity (filled squares) and immunoactivity (open squares). The arrows indicate the beginning of the salt gradient and elution position of the glucocorticoid receptor peak, respectively.

DISCUSSION

The issue of the non-activated glucocorticoid receptor and its physicochemical nature has attracted a great deal of interest during the past several years. However, it has only been possible to trace the glucocorticoid receptor using a radioactive ligand, which simultaneously provokes its activation [4, 5] and consequently a change of the receptor from its non-activated state. As mentioned in the Introduction, the use of sodium molybdate, a potent inhibitor of the activation of glucocorticoid receptor and other steroid receptors [6–13, 17, 18] has helped to partly overcome these difficulties.

Using an ELISA technique based on polyclonal antibodies raised against the purified rat liver cytosolic glucocorticoid receptor [32], we were able to assay the receptor without the use of any labelled hormone-ligand. This technique thus enabled us to avoid the risk of receptor activation described in previous reports [4, 30, 31]. The data obtained in the absence of molybdate revealed that the non-liganded glucocorticoid receptor has a Stokes radius of 6 nm, i.e. the same radius as liganded receptor analyzed under the same conditions. However, when sodium molybdate was introduced into the preparation medium, the glucocorticoid receptor Stokes radius measured was 8 nm. This molybdate dependent behaviour of non-liganded glucocorticoid receptor

was also observed for the liganded glucocorticoid receptor.

In a previous study [25] we have demonstrated that highly purified, activated glucocorticoid receptor has the same structural characteristics as described in this paper for the non-activated, non-liganded form obtained in the absence of molybdate. We, in collaboration with others, have also shown that this purified receptor form binds to glucocorticoid responsive mammary tumour virus (MTV) DNA with a high affinity and a high specificity [46, 47]. In the present investigation we could not detect any association (oligomerization) tendency of highly purified activated glucocorticoid receptor in the presence of molybdate. Yet, we have observed that under low salt concentration conditions (50 mM NaCl) even the activated and highly purified glucocorticoid receptor to some extent (about 20% of hormone bound activity) sediments around 9 S on sucrose gradients while about 80% of the rest of the hormone bound activity sediments around 4 S (unpublished observation). This observation is in accordance with the previously reported data on the stabilization of an oligomeric form of the glucocorticoid receptor in crude preparations under hypotonic conditions and in the presence of leupeptin [14, 15, 19, 24]. However, the purified glucocorticoid receptor also has a tendency to form large aggregates of undefined size which sediment to the bottom of density gradients during sedimentational analysis. Furthermore, visualization of purified glucocorticoid receptor by electron microscopy, either bound or not bound to (MTV) DNA, has shown the existence of a glucocorticoid receptor oligomer of defined size, presumably a tetramer [47].

We propose the following interpretation of the data described above: (i) The glucocorticoid receptor could be an oligomer, both in its non-activated and in its activated state. (ii) The non-activated glucocorticoid receptor oligomer is stabilized by molybdate; the molecular mechanism behind this stabilization as well as the concomitant and presumably linked inhibition of glucocorticoid receptor activation is yet unclear. (iii) The non-activated oligomeric glucocorticoid receptor in the absence of molybdate as well as the activated oligomeric glucocorticoid receptor in the presence or absence of molybdate can be easily dissociated, especially at high ionic strength, and will thus appear as a monomer with a Stokes radius of 6 nm during gel filtration chromatography. (iv) The self aggregational properties of the purified activated glucocorticoid receptor to an oligomer of defined size (9 S peak on glycerol gradients containing 50 mM NaCl, unpublished observation) may be more easily seen with purified receptor preparations where interference from other proteins is minimized.

The proposed oligomeric state of glucocorticoid receptor *in vivo* is in agreement with what has been shown for other DNA-binding regulatory proteins such as the lambda repressor [48], the lac repressor [49] and with what has been proposed for the large T-antigen expressed in SV 40 infected cells [50]. It should be emphasized, however, that the data presented above may be interpreted in other, alternative ways. Furthermore, we have recently described [51, 52] that a non-hormone binding protein interacting with the receptor *in vitro* may stimulate the specific DNA binding properties of the glucocorticoid receptor. This suggests that the native glucocorticoid receptor may contain more than one type of subunit.

In conclusion, the data presented in this report suggest that dissociation of an 8 nm glucocorticoid receptor form to a 6 nm form is not the sole molecular event required for glucocorticoid receptor activation. Indeed, it remains to be shown what role, if any, dissociation of a receptor oligomer to a receptor monomer plays in the process of glucocorticoid receptor activation. Further studies are needed to reveal the subunit structure of the native glucocorticoid receptor in different functional states.

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