Interaction of rat liver glucocorticoid receptor with adenosine 5'-triphosphate

CHARACTERIZATION OF INTERACTION BY USE OF ATP-SEPHAROSE AFFINITY CHROMATOGRAPHY

Virinder K. MOUDGIL and Judith K. JOHN

Department of Biological Sciences, Oakland University, Rochester, MI 48063, U.S.A.

(Received 25 February 1980)

An interaction between rat liver glucocorticoid–receptor complex and immobilized ATP was identified. Rat liver cytosol preparations were incubated with [^3H]triamcinolone acetone for 4 h at 4°C and partially purified by precipitation with $(\text{NH}_4)_2\text{SO}_4$ before use. The resulting glucocorticoid–receptor complex could be selectively adsorbed on to columns of ATP–Sephadex. The freshly prepared cytosol [^3H]triamcinolone acetone–receptor complex had very little affinity for binding to the ATP–Sephadex column, but acquired this ability on temperature- or salt-activation. The presence of 10 mM-sodium molybdate during this salt- or temperature-dependent activation blocked the binding of the receptor complex to ATP–Sephadex. The interaction is reversible, since it can be disrupted by high-salt conditions. A competitive binding assay, using free nucleotides in samples to be chromatographed, revealed a preferential interaction between ATP and the glucocorticoid–receptor complex. Buffer containing ATP was also used to elute the glucocorticoid–receptor complex from ATP–Sephadex columns successfully. When ATP was added to the preparations containing [^3H]triamcinolone acetone–receptor complexes, the steroid specificity or sedimentation properties of the complex remained unaltered. Our results demonstrate an interaction between rat liver glucocorticoid–receptor complex and immobilized ATP and suggest a role of this nucleotide in receptor function.

A two-step mechanism of steroid-hormone action has been widely suggested. On entering a cell, a steroid hormone binds to its specific receptor protein, and the resulting complex then migrates into the cell nucleus, where it is thought to act as a modulator of gene expression (Jensen & DeSombre, 1972; King & Mainwaring, 1974). The actual biochemical function of the receptor, however, has remained unknown. Insufficient information about the regulation of gene activity in higher organisms and the non-availability of sufficiently purified receptor preparations have limited the understanding of steroid-hormone action.

Various approaches have been applied to gain insight into the function of steroid receptors, including studies on their ability to interact with various cellular constituents (Schrader *et al.*, 1972). An interaction between avian progesterone receptor and ATP has been demonstrated (Moudgil & Toft, 1975, 1977). This interaction was demonstrated by the use of ATP–Sephadex affinity chromatography

and was shown to be a reversible process, with a preference for ATP among the nucleotides tested. Although the exact role of ATP in receptor function has remained unclear, increasing evidence indicates that ATP may be influencing various properties of the steroid receptors (Ishii & Aronow, 1973; Munck & Brinck-Johnsen, 1968). ATP has also been reported to stabilize the receptor, and phosphorylation and dephosphorylation mechanisms have been suggested to influence the steroid-binding capacity of glucocorticoid receptor (Nielsen *et al.*, 1977a,b; Sando *et al.*, 1977). In the present studies, we have examined whether these effects involve an interaction between glucocorticoid receptor and ATP.

Materials and methods

All the reagents used were of analytical grade. Nucleotides, activated charcoal, monothioglycerol, unlabelled triamcinolone acetone, cortisol, EDTA, glycerol, CNBr, acrylamide and *NN'*-

methylenebisacrylamide, ammonium persulphate $[(\text{NH}_4)_2\text{S}_2\text{O}_8]$ and $\text{NNN}'\text{N}'$ -tetramethylethylenediamine were purchased from Sigma Chemical Co. Sepharose 4B and Dextran T-70 were from Pharmacia; $[6,7(\text{n})\text{-}^3\text{H}]$ triamcinolone acetonide (30–50 Ci/mmol) was from New England Nuclear, and was repurified by t.l.c. by using methylene chloride/methanol (24:1, v/v). Sodium molybdate was purchased from Merck.

Buffers

Buffer A contained 10 mM-Tris/HCl, 50 mM-monoethioglycerol, 2.5 mM-EDTA, 0.01 M-KCl and 20% (v/v) glycerol, pH 8.0 (at 23°C). Buffer B was the same as buffer A but contained 1 M-KCl.

Preparation of ATP-Sepharose

ATP was covalently linked to Sepharose 4B as previously described (Moudgil & Toft, 1975, 1977) by the method of Lamed *et al.* (1973). In this procedure, adipic acid dihydrazide was attached to CNBr-activated Sepharose. ATP was oxidized by NaIO_4 and then linked to Sepharose hydrazide. The ribose of ATP was therefore attached to Sepharose through the C_6 bridge of adipic acid dihydrazide. Our preparations contained 5–7 μmol of ATP/ml of packed resin as determined by phosphate analysis (Moudgil & Toft, 1975; King, 1932).

Preparation of glucocorticoid receptor

Male albino rats of Yale strain (150–200 g) were obtained from Maguran Farms (Troy, MI, U.S.A.). The rats were bilaterally adrenalectomized and kept on salt water (0.9% NaCl) for 3 days, and were then killed by cervical dislocation. The whole liver tissue was quickly excised, and rinsed in cold 0.9% NaCl to remove any adhering blood. The tissue was minced and homogenized in 2 vol. (v/w) of homogenization buffer (40 mM-Tris/HCl, 50 mM-monoethioglycerol, 10% glycerol and 0.01 M-KCl, pH 8.0) with a Tissuemizer (Tekmar model SDT). The homogenate was first centrifuged at 12000 g for 10 min and then at 150000 g for 1 h. The resultant clear supernatant was used immediately for various assays. For some experiments the fresh cytosol was incubated with $[^3\text{H}]$ triamcinolone acetonide for 4 h at 4°C and fractionated by 50%-satd. $(\text{NH}_4)_2\text{SO}_4$. The resultant precipitate, containing the glucocorticoid–receptor complex, was used immediately or stored at –85°C for 1 week without any loss of steroid from the complex. Because of its known interference with the process of receptor activation (Jensen *et al.*, 1973), EDTA was omitted in experiments dealing with activation of cytosolic receptor complexes.

Sucrose-density-gradient analysis

Linear 5–20% sucrose gradients (4.4 ml) were

prepared in buffer containing 10 mM-Tris/HCl, 50 mM-monoethioglycerol and 0.3 M-KCl by using a Beckman gradient former. Samples (0.2 ml) containing glucocorticoid–receptor complex with and without additives (e.g. ATP) were layered on the gradients. When appropriate, the samples were diluted with 10 mM-Tris/HCl buffer, pH 8.0, to lower the glycerol concentration. The gradients were centrifuged at 150000 g on a Sorvall OTD-65 ultracentrifuge for 4 h in a TV-865 vertical rotor. The tubes were pierced at the bottom and 0.2 ml fractions collected. $[^{14}\text{C}]$ Ovalbumin was used as a marker protein (Rice & Means, 1971) and layered on a separate gradient as described previously (Moudgil & Toft, 1975, 1977).

Steroid-binding assays

Charcoal-adsorption assays were performed to monitor total and non-specific steroid binding. Duplicate or triplicate samples of receptor preparations were incubated with 10 nM- $[^3\text{H}]$ triamcinolone acetonide. To determine the non-specific binding, a 100–500-fold excess of unlabelled steroid (cortisol or triamcinolone acetonide) was added to other duplicate samples before the addition of labelled steroid. In cases where complexes were already formed, non-specific binding was determined by heating the samples at 60°C for 30 min in the presence of excess unlabelled steroid to destroy specific receptors (Buller *et al.*, 1976). An equal volume of Dextran-coated charcoal (1% Norit A and 0.05% Dextran T-70, made in 10 mM-Tris/HCl/12 mM-thioglycerol, pH 8.0) was added to each tube and the contents were mixed gently. After 5 min incubation at 4°C, tubes were centrifuged at 1000 g for 5–10 min and the supernatants used to measure radioactivity. The specific binding represented the total radioactivity minus the values from heated or unlabelled hormone-treated samples. Less than 8% of the total binding was observed to be non-specific.

Affinity chromatography

ATP-Sepharose affinity chromatography was performed essentially as described previously (Moudgil & Toft, 1975, 1977). Small columns containing 2 ml of packed ATP-Sepharose were thoroughly washed and equilibrated with buffer A. The samples were layered on the columns, washed with 20 vol. of buffer A and the complexes were eluted from the affinity columns by using buffer B. The specific details are given in the Figure legends.

Polyacrylamide-gel electrophoresis

Electrophoresis was performed by the method of Miller *et al.* (1975), with small modifications. The gels (5% acrylamide; 7.5 cm long $\times$ 0.6 cm diameter) were prepared at room temperature and contained 3% cross-linking. Before a sample was run, mercap-

Fig. 1. *Chromatography of glucocorticoid-receptor complex on ATP-Sepharose*

(a) An $(\text{NH}_4)_2\text{SO}_4$ -fractionated pellet containing $[^3\text{H}]\text{triamcinolone acetoneide-receptor complex}$ was dissolved in 1 ml of 10 mM-Tris/HCl, pH 8.0, and cleared by centrifugation at 12000 *g* for 10 min. The clear supernatant was treated with charcoal suspension to remove the free steroid. A 1.1 ml sample was then chromatographed over a 2 ml ATP-Sepharose column pre-equilibrated with buffer A (see the Materials and methods section). The column was washed with the same buffer and the adsorbed complexes were eluted with buffer B. Ten 1.4 ml fractions were collected each with low- and high-salt buffers at a rate of 0.45 ml/min; 0.5 ml samples of each were taken and scintillation fluid (5 ml) was added to measure the radioactivity. The first peak of radioactivity represents the unadsorbed receptor complex. (b) Salt-gradient elution of $[^3\text{H}]\text{triamcinolone acetoneide-receptor complex}$ from the ATP-Sepharose column. A 1.2 ml sample of receptor as prepared in Fig. 1(a) was layered on a 5 ml ATP-Sepharose column pre-equilibrated with buffer A. After the sample had entered the resin, it was

toacetic acid (0.05 ml of 0.1 M-mercaptoacetic acid in 45% glycerol) was applied to the gels, followed by the sample (0.05 ml) in 20% glycerol. The gels and reservoir buffers contained 10% glycerol. A 0.01% solution (10 μl) of Bromophenol Blue in 15% glycerol was also layered on the gel as an electrophoretic marker. Electrophoresis was carried out at 4°C for about 4 h at 3 mA/gel. One set of gels was stained for 1 h with 0.1% Coomassie Blue in 7% acetic acid. A duplicate set of gels was sliced into 2 mm sections for determining the migration of $[^3\text{H}]\text{triamcinolone acetoneide-receptor complex}$. Each gel section was transferred to a vial containing 5 ml of scintillation fluid (Beckman BioSolv BBS-3/Spectrafluor PPO-POPOP/toluene, 2:1:15, by vol.).

Radioactivity in samples was determined by combining aqueous samples with 5 ml of scintillation fluid consisting of 4.2% (v/v) Spectrafluor (Amersham) in toluene. The protein concentration in various samples was determined by the method of Bradford (1976).

Results

Fig. 1 illustrates a typical profile of the chromatography of glucocorticoid-receptor complex on columns of ATP-Sepharose. Before chromatography, rat liver cytosol was labelled with $[^3\text{H}]\text{triamcinolone acetoneide}$ for 4 h at 4°C and was then fractionated with 50%-satd. $(\text{NH}_4)_2\text{SO}_4$ prepared in 10 mM-Tris/HCl, pH 8.0. The resuspended receptor complex was then applied to the ATP-Sepharose column. As shown in Fig. 1(a), most of the complex was adsorbed to the affinity resin and could be eluted with buffers containing 1 M-KCl. All the buffers used in column chromatography contained 20% glycerol, which helped to stabilize the glucocorticoid-receptor complex. The presence of glycerol was found to increase the half-life of the $[^3\text{H}]\text{triamcinolone acetoneide-receptor complex}$ at 23°C (results not shown). The amount of gluco-

washed with buffer A and ten 1.5 ml fractions were collected. At this point, the adsorbed receptor-complex was eluted with a linear salt gradient (—) made from 30 ml each of buffers A and B; 1.5 ml fractions were collected and 1 ml portions of each were used for measurement of radioactivity. Small samples were used to determine the protein concentration in each fraction (Bradford, 1976). The peak fractions eluted with 1 M-KCl accounted for 52% of total specific steroid binding present before chromatography. O, Protein concn.; ●, specific $[^3\text{H}]\text{glucocorticoid binding}$.

corticoid-receptor complex adsorbed to the ATP-Sepharose column varied between 40 and 95%, with an average adsorption of 50%, compared with the total receptor complex present in the sample before chromatography. The first peak of radioactivity represents the free steroid plus the unadsorbed glucocorticoid-receptor complex. The second peak of radioactivity represents the specific glucocorticoid-receptor complex, since free [^3H]triamcinolone acetonide and receptor complexes formed in the presence of excess unlabelled triamcinolone acetonide or cortisol were not retained on the ATP-Sepharose column. The excess cortisol or triamcinolone acetonide eliminated the saturable binding of [^3H]triamcinolone acetonide to receptors and the retention of [^3H]triamcinolone that was unbound or bound in a non-saturable manner. More than 90% of the applied [^3H]triamcinolone acetonide was routinely recovered from these columns, and more than 70% of the hormone bound in the original sample could be accounted for in the fractions eluted with 1 M-KCl. When a small sample from the peak fraction eluted with 1 M-KCl was passed through a column of Sephadex G-75, almost all the radioactivity was protein-bound and appeared in the void volume (results not shown).

Although ATP-Sepharose affinity chromatography provides a very effective method for studying the receptor interaction, it is also a useful technique for receptor purification. Before chromatography, the receptor samples were routinely assayed for both protein concentration and amount of receptor-bound hormone. The stepwise elution (Fig. 1a) allowed a purification of about 40-fold. However, a significant improvement in purification was achieved by using salt-gradient elution. Fig. 1(b) illustrates a salt-gradient (0.1–1 M-KCl) elution of glucocorticoid-receptor complex from an ATP-Sepharose column. A single peak of bound [^3H]triamcinolone acetonide was eluted at 0.5–0.6 M-KCl. Although the appearance of this broad peak indicated some heterogeneity, the low-salt and high-salt sides of this peak yielded similar glucocorticoid receptor on further characterization by rechromatography on ATP-Sepharose, analysis on sucrose gradients and steroid-competition studies. This result indicates that the binding of ATP to the receptor is of high affinity and also suggests a homogeneity of the receptor in its interaction with ATP-Sepharose. This procedure provides at least 100-fold purification, with a receptor recovery of 60%. In some experiments the peak fractions accounted for a purification of 200–300-fold.

Different receptor preparations were also analysed by polyacrylamide-gel electrophoresis (Fig. 2). The gels contained 50 mM-monothioglycerol and 20% glycerol, which appeared to stabilize the receptor. A portion (10–20%) of the glucocorti-

coid-receptor complex from cytosol, $(\text{NH}_4)_2\text{SO}_4$ -fractionated or ATP-Sepharose-purified preparation entered the gel and migrated as a single band. ATP-Sepharose chromatography did not alter the electrophoretic behaviour of the receptor, although it removed many of the protein bands detected in the crude receptor preparations.

The integrity of the [^3H]triamcinolone acetonide-receptor complex after affinity chromatography was tested by various criteria. When sucrose-gradient centrifugation was employed, the receptor complex sedimented as a 4S entity in

Fig. 2. Polyacrylamide-gel electrophoresis of different rat liver preparations containing glucocorticoid-receptor complex

Samples containing an equal amount of protein from $(\text{NH}_4)_2\text{SO}_4$ -fractionated and ATP-Sepharose-purified preparations were layered over 5% acrylamide gels containing 20% glycerol and 3% cross-linking. A 0.01% solution of Bromophenol Blue in 15% glycerol was also layered on a separate gel as an electrophoretic marker. Electrophoresis was carried out at 4°C for about 4 h at 3 mA/gel. One set of gels was stained for 1 h with 0.1% Coomassie Blue in 7% acetic acid (results not shown). A duplicate set of gels was sliced into 2 mm sections for determining the migration of [^3H]triamcinolone acetonide-receptor complex. ●, $(\text{NH}_4)_2\text{SO}_4$ -fractionated [^3H]triamcinolone acetonide-receptor complex; ○, ATP-Sepharose-purified receptor complex.

high-salt environment both before and after affinity chromatography (Fig. 3). A tendency for the $(\text{NH}_4)_2\text{SO}_4$ -fractionated receptor to aggregate at low ionic strength has been described previously (Schrader *et al.*, 1972; Moudgil & Toft, 1975; Toft *et al.*, 1976b), but we found little or no such aggregation of crude or purified glucocorticoid-receptor complexes in low-salt-containing sucrose gradients, which yielded a 4S component (results not shown). By using a competitive-displacement binding assay, the steroid-binding specificity of the purified receptor was also tested. The receptor in the cytosol preparation was incubated with various steroids before the addition of $[\text{H}]$ triamcinolone acetonide, whereas the complexes eluted from the

ATP-Sepharose columns were incubated with the same steroids at 23°C. There was, however, a slight loss (15–25%) of the steroid-binding capacity of glucocorticoid receptor at 23°C. Fig. 4 illustrates the steroid-binding specificity of the crude and the ATP-Sepharose-purified receptor. Only 50% inhibition of ATP-Sepharose-purified receptor binding was given by 100-fold excess cortisol, because the receptor in this case was already complexed to $[\text{H}]$ triamcinolone acetonide, which accounted for a slower exchange between the steroids. Similar results were obtained by using triamcinolone acetonide as a competitor. Clearly, the studies presented here involved a specific glucocorticoid receptor, which

Fig. 3. Sucrose-density-gradient-centrifugation analysis of the crude and ATP-Sepharose-purified receptor fractions

The freshly prepared cytosol was incubated with 10 nM $[\text{H}]$ triamcinolone acetonide and the preparation fractionated with 50% satd. $(\text{NH}_4)_2\text{SO}_4$. The resultant pellet was redissolved in 10 mM-Tris/HCl, pH 8.0. A portion (200 μ l) from the above sample and an equal volume from the peak fraction eluted with 1 M-KCl from an ATP-Sepharose column were layered on separate linear sucrose gradients (5–20%) containing 10 mM-Tris/HCl/12 mM-thioglycerol/0.3 M-KCl, pH 8.0. The column fraction was diluted with 10 mM-Tris/HCl, pH 8.0, to lower the glycerol concentration. The gradients were centrifuged for 4 h at 4°C at 150 000 g in a Sorvall OTD-65 ultracentrifuge in a TV-865 vertical rotor. Samples containing $[\text{H}]$ triamcinolone acetonide-receptor complexes without and with 0.1–5 mM-ATP were also used to determine the effects of nucleotide on the sedimentation rate. After the run, 0.22 ml fractions were collected by piercing the bottom of the tubes. $[\text{H}]$ Ovalbumin was layered on a separate gradient and served as a marker (3.7S) (Rice & Means, 1971). ●, $(\text{NH}_4)_2\text{SO}_4$ -fractionated receptor complex; ○, ATP-Sepharose-purified receptor complex.

Fig. 4. Steroid-binding specificity of $[\text{H}]$ triamcinolone acetonide-receptor complex in crude and semi-purified preparations

Different steroids were preincubated (100-fold excess) with the cytosol preparations before the addition of 8 nM $[\text{H}]$ triamcinolone acetonide. With ATP-Sepharose-purified preparations, the different steroids were included in a series of samples and incubated at 23°C for 2 h to allow an exchange. After this, the charcoal-adsorption assay was performed and radioactivity measured as detailed in the Materials and methods section. □, Crude receptor; ▨, ATP-Sepharose-purified receptor; C, control; F, cortisol; P, progesterone; E, oestradiol; T, dihydrotestosterone. Results are means $\pm$ S.E.M. of triplicate determinations in a single experiment. Each experiment was repeated three to six times with identical results.

retained its steroid-binding specificity after the affinity chromatography.

ATP is a necessary constituent of this affinity chromatography. In our procedure, ATP was covalently linked to Sepharose 4B via a C_6 spacer, adipic acid dihydrazide. This method is relatively simple, and unchanged hydrazide groups on the Sepharose do not have ion-exchange properties at neutral or high pH (Lamed *et al.*, 1973). Fig. 5 demonstrates that, under the salt conditions normally employed, the receptor complex had little or no affinity for Sepharose 4B or Sepharose hydrazide (an intermediate in the preparation of ATP-Sepharose). The selective adsorption of the glucocorticoid-receptor complex was evident only when ATP was linked to Sepharose.

To demonstrate that the retention of the glucocorticoid-receptor complex on the affinity resin was due to an interaction between receptor and ATP, the following experiments were performed. Two receptor samples were chromatographed on two identical ATP-Sepharose columns, except that one sample was treated with 5 mM-ATP for 30 min. The presence of this free nucleotide would competitively decrease the binding of receptor complex to ATP-Sepharose. Fig. 6 demonstrates that the decreased receptor-complex binding to the ATP-Sepharose column in the presence of free ATP is indicative of an interaction between the nucleotide and the glucocorticoid-receptor complex. The use of higher concentrations of ATP in samples to be chromatographed caused a comparable decrease in recep-

tor-complex binding to ATP-Sepharose columns. Our ATP-Sepharose preparations contained 5–7 mM-ATP and required about 50 mM free ATP to block completely receptor-complex binding to affinity columns.

The specificity of this nucleotide-receptor-complex interaction was tested by using the above competition assay, by incubating different nucleotides (5 mM) with the receptor-complex samples to be chromatographed. Identical ATP-Sepharose columns were used simultaneously to avoid time-dependent quantitative differences in the recovery of complexes. The binding of nucleotides to the receptor complex was judged by their ability to decrease the adsorption of receptor complexes to ATP-Sepharose columns. The results of several different experiments are presented in Table 1. Under the conditions employed in these competitive binding assays, only ATP appeared to be an effective competitor. These results clearly indicate a preference for ATP over the other nucleotides tested. These observations, however, do not rule out the possibility that there may be different nucleotide-binding sites, which may explain why other nucleotides did not compete with ATP-binding site(s) of the receptor.

The cytosol glucocorticoid-receptor complex has very little affinity for ATP-Sepharose. However, it

Fig. 5. Analysis of the ligand specificity in the ATP-Sepharose affinity chromatography. $(NH_4)_2SO_4$ -fractionated receptor complex was chromatographed on three identical-sized (2 ml) columns made with Sepharose alone ($\bullet$), Sepharose hydrazide (Δ) and ATP-Sepharose ($\circ$). The conditions of the chromatography assay were the same as in Fig. 1.

Fig. 6. Determination of the specificity of ATP-Sepharose interaction with glucocorticoid-receptor complex. An $(NH_4)_2SO_4$ -fractionated receptor-complex pellet was redissolved in 1 ml of buffer A and divided into three equal portions. Cortisol (1 mM) was added to one portion and the mixture was heated for 60 min at 60°C ($\circ$). The second sample was incubated with 0.2 mM-ATP for 30 min at 4°C (Δ) and the third sample served as a control ($\bullet$). All three samples were chromatographed separately on 2 ml ATP-Sepharose columns. In the ATP-treated sample, the total radioactivity in fractions eluted with 1 M-KCl was 48% of the control.

Table 1. Competition by free nucleotides with immobilized ATP

The results are expressed as the mean $\pm$ S.E.M. of the percentage of total radioactivity recovered from the fractions eluted with 1 M-KCl from three or six different experiments.

Nucleotide (5 mM)	ATP-Sephadex-bound [³ H]triamcinolone acetonide (% of control)	No. of experiments
ATP	49 $\pm$ 11	6
CTP	96 $\pm$ 3.5	3
UTP	87 $\pm$ 13	3
GTP	96 $\pm$ 4.0	3
ADP	94 $\pm$ 5.5	3
AMP	80 $\pm$ 7	3
Cyclic AMP	94 $\pm$ 6	3
p[NH]ppA	88 $\pm$ 11	3

Fig. 7. Requirement for heat-activation as a requisite for receptor-complex binding to ATP-Sephadex
A series of tubes containing cytosol [³H]triamcinolone-receptor complex were set up and the complex was activated by incubating the tubes at 23°C for various time periods shown (○, 60 min; ●—●, 40 min; △, 30 min; ▲, 15 min; ●---●, control). After this the samples were chromatographed on identical 2 ml ATP-Sephadex columns as described in Fig. 1; 1 ml portions were used from each fraction (1.4 ml) to measure radioactivity.

acquired this ability to bind to the ATP-Sephadex column when the cytosol receptor-complex preparations were either warmed at 23°C or treated with high salt (0.2 M-KCl). All the experiments presented above utilized (NH₄)₂SO₄-fractionated receptor complex, which readily binds to ATP-

Table 2. Effect of salt activation on the binding of glucocorticoid-receptor complex to ATP-Sephadex

The cytosol samples containing [³H]triamcinolone acetonide-receptor complex were treated with increasing salt concentrations (0.01–0.3 M-KCl) for 30 min and the mixtures were diluted with buffer A (1:1, v/v) before their chromatography on separate 2 ml ATP-Sephadex columns as described in Figs. 1 and 7. The peak fractions eluted with 1 M-KCl were pooled and small portions (0.5 ml) were used to measure the radioactivity.

KCl concn. (M)	10 ⁻³ × ATP-Sephadex-bound [³ H]triamcinolone-receptor complex (c.p.m.)
0.01	2.06
0.1	3.49
0.2	4.40
0.3	2.85

Sephadex. In contrast with freshly prepared cytosol, the (NH₄)₂SO₄-fractionated receptor complex is in the activated state in that it binds to isolated nuclei at 4°C (Buller *et al.*, 1976). The results in Fig. 7 indicate that activation of the receptor complex is a prerequisite for its binding to ATP-Sephadex. Less than 20% of the total cytosolic receptor complex present before chromatography binds to ATP-Sephadex columns. The receptor-complex binding to the ATP-Sephadex column markedly increased with preincubation at 23°C, and under optimum conditions (40 min at 23°C) about 70% of the total receptor complex bound to the column. The increased ATP binding observed in the cytosol receptor samples not treated at 23°C is probably due to a partial activation of the receptor complex under the assay conditions used. We also used salt treatment as a procedure for receptor activation. The results in Table 2 indicate that the salt treatment of the cytosol glucocorticoid-receptor complex also increased ATP binding. This increased ATP binding observed after heat- or salt-activation could not be

accounted for by a change in hormone-binding activity as determined by the charcoal adsorption assay (results not shown).

Toft *et al.* (1976a) and Miller & Toft (1978) had previously observed similar activation-dependent binding of the avian progesterone receptor to ATP-Sepharose. Although nucleotide-glucocorticoid-receptor interaction has the same characteristics, there were, however, subtle differences. In contrast with observations with progesterone receptor, we found higher ATP binding by receptor complex when the latter was activated by temperature elevation. Also, the time required for maximal activation and subsequent receptor-complex binding to ATP was 40 min, compared with 60 min reported for progesterone receptor (Miller & Toft, 1978). The discrepancies between the two systems stem from the known labile nature of the glucocorticoid receptor (Sando *et al.*, 1977). These results demonstrate that the binding to ATP-Sepharose is a property of an activated receptor complex, and this may be a general phenomenon of other receptor systems (Miller & Toft, 1978).

Nielsen *et al.* (1977a,b) have shown that the addition of molybdate stabilizes the glucocorticoid receptor from rat thymocytes. It has been further shown that the addition of molybdate blocks the heat-induced activation of the avian progesterone-receptor molecule (Nishigori & Toft, 1980; Toft *et al.*, 1979). In these studies cytosol was incubated with 50 mM-molybdate for 30 min before receptor activation, and the extent of receptor activation was measured by observing receptor binding to ATP-Sepharose. The presence of molybdate blocked the receptor binding to ATP-Sepharose when it was added before receptor activation. However, molybdate did not directly block receptor binding to ATP-Sepharose, since it was not effective when added after receptor activation. It is evident that molybdate blocked the activation process itself. Our results are consistent with those previously observed (Toft *et al.*, 1979; Nishigori & Toft, 1980; Moudgil *et al.*, 1980). When receptor samples were activated by warming at 23°C for 40–60 min, most of the receptor complex was adsorbed on an ATP-Sepharose column. Pretreatment with 10 mM-molybdate almost completely blocked the ability of the receptor complex to bind to ATP-Sepharose. The presence of molybdate had little or not effect on the binding of glucocorticoid-receptor complex when $(\text{NH}_4)_2\text{SO}_4$ -fractionated receptor preparations were used which contained an activated form of glucocorticoid receptor (results not shown).

The results of the preceding experiments were indicative of a possible use of ATP as an effective eluent in this affinity chromatography. Our efforts to elute the receptor complex from the ATP-Sepharose column with 10 mM-Tris/HCl buffer con-

taining 50 mM-ATP were successful. More than 70% of the receptor complex applied to the column could be recovered by elution with 50 mM-ATP. A subsequent elution with 1 M-KCl did not remove any significant amount of receptor complex, and the recovery and purification of receptor by this method were comparable with the results obtained by salt elution. The ATP solution was prepared in 10 mM-Tris/HCl buffer and its pH was adjusted to 8.0. The presence of this high concentration of ATP did not affect the steroid-binding properties under these assay conditions (results not shown).

Since ATP binding could be of functional significance to the steroid-hormone action, we attempted to determine if the presence of steroid influenced this interaction. Our attempts to date have resulted in little success, possibly because of the highly unstable nature of the glucocorticoid receptor in the absence of steroid. Receptor fractionated with $(\text{NH}_4)_2\text{SO}_4$ in the absence of steroid immediately became inactive and therefore posed problems in its use for ATP-Sepharose chromatography. However, progesterone receptor fractionated with $(\text{NH}_4)_2\text{SO}_4$ in the absence of steroid has been shown to retain its ability to bind to ATP-Sepharose (Moudgil & Toft, 1977).

The presence of the nucleotide did not cause any alteration in the steroid-binding properties of the glucocorticoid receptor (results not shown). $(\text{NH}_4)_2\text{SO}_4$ -fractionated receptor was redissolved in 10 mM-Tris/HCl, pH 8.0, and a series of receptor samples was incubated with 10 mM-[^3H]triamcinolone acetonide for 4 h at 4°C. After this, different volumes of concentrated ATP stock solution were added. The extent of hormone binding was determined by the charcoal-adsorption assay.

Discussion

Since its initial identification (Moudgil & Toft, 1975), the interaction of progesterone receptor with ATP has been the object of many investigations (Moudgil & Toft, 1976, 1977; Toft *et al.*, 1976a,b, 1977, 1979; Miller & Toft, 1978; Nishigori *et al.*, 1978; Moudgil *et al.*, 1980). In the present report we describe yet another receptor system exhibiting interaction with immobilized ATP. The glucocorticoid-receptor-complex binding to ATP-Sepharose is reversible, ionic in nature and shows a preference for ATP over the other nucleoside triphosphates, AMP and cyclic AMP. Owing to its selective nature and the remarkable yield of the adsorbed receptor, this interaction offers an attractive procedure for receptor purification.

As demonstrated in Fig. 7 and Table 2, ATP-Sepharose binding is a characteristic of the activated form of glucocorticoid-receptor complex. Freshly prepared cytosol receptor complex exhibits

little binding to ATP-Sepharose. The binding was greatly enhanced after its activation by salt treatment or temperature elevation. This ability of ATP-Sepharose to bind activated receptor complex provides an efficient tool by which to characterize or study the process of receptor activation. Also, this affinity chromatography can be used to distinguish between an activated and a non-activated form of the glucocorticoid-receptor complex. The results for avian progesterone receptor (Moudgil & Toft, 1975, 1977; Miller & Toft, 1978) and oestradiol receptor from rat uterus (Miller & Toft, 1978) and hen oviduct (Moudgil & Weekes, 1978), together with the present findings on the glucocorticoid receptor, suggest that ATP binding might be a property of activated steroid receptors in general.

Similarities between ATP binding by the glucocorticoid-receptor complex and the known interaction of the steroid receptor with phosphate (phosphocellulose) and with polynucleotides such as DNA have been considered. Although some relationship may exist between these interactions, there are clear differences. The salt concentrations generally required to disrupt ATP binding by glucocorticoid-receptor complex are much higher (0.5–0.6 M-KCl) than those required to disrupt receptor binding to phosphocellulose or DNA-cellulose.

The actual role of ATP in receptor function is not clear, although our results suggest that ATP may be biologically important in a function of the activated receptors (John & Moudgil, 1979). ATP either may be involved in the process of activation itself or may influence the function of the receptor at sites on nuclear chromatin. In this regard, nucleotides have been shown to inhibit or accelerate the rate of progesterone-receptor-complex activation, depending on the concentration used (Toft *et al.*, 1976a, 1977). Low nucleotide concentrations (<1 mM) inhibited, whereas higher ATP concentrations (>1 mM) stimulated the rate of receptor activation. The possibility that ATP binding may stabilize the receptor during the activation process has been discussed previously (Miller & Toft, 1978).

In order to get more insight into the role of ATP in receptor function, different inhibitors of this interaction have been sought, namely *o*-phenanthroline, rifamycin AF/013, pyridoxal 5'-phosphate and aurointricarboxylic acid (Moudgil & Toft, 1976; Toft *et al.*, 1976a, 1977; Nishigori *et al.*, 1978; Moudgil & Weekes, 1978; Moudgil *et al.*, 1978, 1980; Moudgil & Eessalu, 1980). These inhibitors block the binding of the steroid receptor to ATP-Sepharose and isolated nuclei, suggesting that the nucleotide-binding site(s) on the receptor may be closely associated with the region(s) that participate(s) in nuclear binding process. Since receptor uptake by nuclei and binding to ATP are different processes, it is possible that two separate inter-

actions are involved in these processes, both of which are altered by these inhibitors. This notion is supported by the fact that nuclear binding can be disrupted by 0.3 M-KCl, whereas much higher salt concentration is needed to disrupt the ATP binding (Fig. 1b).

In addition, several published reports suggest a possible relationship between steroid-hormone receptors and ATP. The glucocorticoid receptor from mouse fibroblasts has been shown to be stabilized by the addition of glucose metabolites and ATP (Ishii & Aronow, 1973). Cellular ATP concentrations have been reported to be critical for rat thymus glucocorticoid-receptor activity (Munck & Brinck-Johnsen, 1968; Bell & Munck, 1973). Also, nuclear binding of the oestradiol receptor has been shown to be enhanced by incubation of nuclei with ATP (King *et al.*, 1971). More recently, an ATP-dependent alteration of L-cell glucocorticoid receptors from a non-steroid-binding form to an active steroid-binding form has been reported (Nielsen *et al.*, 1977a). The specific glucocorticoid-binding capacity in cytosol prepared from L-929 mouse fibroblasts was shown to be inactivated by an endogenous inhibitor with a half-life of approx. 2 h at 25°C. Addition of ATP (5–10 mM) not only slowed down the rate of this inactivation, but also was effective in re-activation of this completely inactivated steroid-binding capacity.

At the present time there are many indications as to the role of ATP in receptor function. A role of ATP as an energy source in either of the two stages of receptor activity has been postulated (Moudgil & Toft, 1975). ATP may help to translocate the receptor complex from a soluble or cytoplasmic phase to sites on nuclear chromatin, or it may function as a regulatory molecule and alter receptor function at the nuclear chromatin level. These postulations are strengthened by the reports (Munck & Brinck-Johnsen, 1968; Bell & Munck, 1973; King & Mainwaring, 1974; Sando *et al.*, 1977; Nielsen *et al.*, 1977a,b) that ATP is involved either directly or indirectly in a process which converts the glucocorticoid receptor from a non-steroid-binding form into a steroid-binding active form (Sando *et al.*, 1977; Nielsen *et al.*, 1977a,b). Our observations (John & Moudgil, 1979; Moudgil & John, 1980) that ATP can itself transform the cytosol glucocorticoid-receptor complex to a nuclear binding form at 4°C and enhance temperature-dependent transformation suggest that energy-requiring step(s) may be required in the translocation of the receptor complex from cytoplasm to nucleus. It is also possible that ATP may act as an energy source for an unknown chemical reaction or as a regulatory molecule causing changes in receptor conformation and exposing site(s) responsible for the nuclear uptake and chromatin binding of glucocorticoid-receptor complex. In any case, evidence has been

presented here which strongly suggests an important role for ATP in receptor function.

We thank Shaun Healy, Tamara Shaffer and Alan Jackson for their help during the initial part of this study. This work is supported by NIH Grant AM-20893-01A1.

References

- Bell, P. A. & Munck, A. (1973) *Biochem. J.* **136**, 97–107
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Buller, R. F., Toft, D. O., Schrader, W. T. & O'Malley, B. W. (1976) *J. Biol. Chem.* **250**, 801–808
- Ishii, D. N. & Aronow, L. (1973) *J. Steroid Biochem.* **4**, 593–603
- Jensen, E. V. & DeSombre, E. R. (1972) *Annu. Rev. Biochem.* **41**, 203–253
- Jensen, E. V., Mohla, S., Brecher, P. I. and DeSombre, E. R. (1973) *Adv. Exp. Med. Biol.* **36**, 60–80
- John, J. K. & Moudgil, V. K. (1979) *Biochem. Biophys. Res. Commun.* **90**, 1242–1248
- King, E. G. (1932) *Biochem. J.* **26**, 292–297
- King, R. J. B. & Mainwaring, W. P. P. (1974) *Steroid-Cell Interactions*, University Park Press, Baltimore
- King, R. J. B., Gordon, J., Marx, J. & Steggle, A. W. (1971) in *Basic Actions of Sex Steroids on Target Organs* (Hubinout, P. O., Leroy, F. & Galand, P., eds.), pp. 21–43, S. Karger, Basel
- Lamed, R., Levin, Y. & Wilcheck, M. (1973) *Biochim. Biophys. Acta* **304**, 231–235
- Miller, J. B. & Toft, D. O. (1978) *Biochemistry* **17**, 173–177
- Miller, L. K., Diaz, S. C. & Sherman, M. R. (1975) *Biochemistry* **14**, 4433–4443
- Moudgil, V. K. & Eessalu, T. E. (1980) *Biochim. Biophys. Acta* **627**, 301–312
- Moudgil, V. K. & John, J. K. (1980) *Biochem. J.* **190**, 799–808
- Moudgil, V. K. & Toft, D. O. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 901–905
- Moudgil, V. K. & Toft, D. O. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3443–3447
- Moudgil, V. K. & Toft, D. O. (1977) *Biochim. Biophys. Acta* **490**, 477–488
- Moudgil, V. K. & Weekes, G. A. (1978) *FEBS Lett.* **94**, 324–326
- Moudgil, V. K., Eessalu, T. E. & Weekes, G. A. (1978) *J. Steroid Biochem.* **9**, 837
- Moudgil, V. K., Nishigori, H., Eessalu, T. E. & Toft, D. O. (1980) in *Gene Regulation by Steroid Hormones* (Roy, A. & Clark, J., eds.), pp. 106–119, Springer-Verlag, New York
- Munck, A. & Brinck-Johnsen, T. (1968) *J. Biol. Chem.* **243**, 5556–5565
- Nielsen, C. J., Sando, J. J., Vogen, W. M. & Pratt, W. B. (1977a) *J. Biol. Chem.* **252**, 7568–7578
- Nielsen, C. J., Vogen, W. M. & Pratt, W. B. (1977b) *Cancer Res.* **37**, 3420–3426
- Nishigori, H. & Toft, D. O. (1980) *Biochemistry* **19**, 77–83
- Nishigori, H., Moudgil, V. K. & Toft, D. O. (1978) *Biochem. Biophys. Res. Commun.* **80**, 112–118
- Rice, R. H. & Means, G. E. (1971) *J. Biol. Chem.* **246**, 831–832
- Sando, J. J., Nielsen, C. J. & Pratt, W. B. (1977) *J. Biol. Chem.* **252**, 7579–7582
- Schrader, W. T., Toft, D. O. & O'Malley, B. W. (1972) *J. Biol. Chem.* **247**, 2401–2407
- Toft, D. O., Lohmar, P. H., Miller, J. B. & Moudgil, V. K. (1976a) *J. Steroid Biochem.* **7**, 1053–1059
- Toft, D. O., Moudgil, V. K. & Lohmar, P. H. (1976b) in *Hormone-Receptor Interactions: Molecular Aspects* (Levey, G. S., ed.), pp. 243–264, Marcel Dekker, New York
- Toft, D. O., Moudgil, V. K., Lohmar, P. H. & Miller, J. B. (1977) *Ann. N.Y. Acad. Sci.* **286**, 29–42
- Toft, D. O., Roberts, P. E., Nishigori, H. & Moudgil, V. K. (1979) in *Steroid Hormone-Receptor Systems* (Leavitt, W. W. & Clark, J. H., eds.), pp. 329–341, Plenum, New York