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THE PROPERTIES AND FUNCTIONAL SIGNIFICANCE OF ATP BINDING TO PROGESTERONE RECEPTORS

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SUMMARY

Affinity chromatography has been used to study the binding of ATP to the avian progesterone receptor. A resin which selectively binds the receptor protein was prepared by linking ATP covalently to Sepharose 4B. Receptor bound to the affinity resin was recovered in a single peak upon gradient elution with ATP or KCl. While affinity chromatography was normally accomplished using the [^{3}H]-progesterone–receptor complex, the hormone was not necessary for ATP binding when the receptor used was first fractionated by ammonium sulfate precipitation. However, further analysis showed that the cytosol receptor must first be activated by heat or high salt treatment before ATP binding can occur. This observation suggests that ATP may be involved in an activity of the receptor during or subsequent to nuclear binding of the receptor complex. This is supported by the finding that two inhibitors, o-phenanthroline and rifamycin AF/013, block the binding of receptor to both isolated nuclei and to ATP-Sepharose.

INTRODUCTION

The avian progesterone receptor has been characterized by purification and physico-chemical analysis [1–3], but the actual biochemical function of this protein remains unknown. A recent report from this laboratory illustrated an interaction between the progesterone receptor and ATP [4]. This interaction was observed by the use of ATP-Sepharose affinity chromatography and was shown to be a reversible process with a preference for ATP among the nucleotides tested. Since the selective nature of this interaction suggests a role for ATP in some aspect of receptor function, a more thorough characterization of the binding of receptor to immobilized ATP was undertaken.

EXPERIMENTAL

All reagents were of analytic grade and were made up in glass-distilled water. Adenosine triphosphate was from Schwarz–Mann; Sepharose 4B and Dextran T-70 from Pharmacia, Uppsala, Sweden; o-phenanthroline from Fischer Scientific; thioglycerol and activated charcoal from Sigma; and, [1,2,^{3}H]-progesterone (50 Ci/mmol) from New England Nuclear. Rifamycin AF/013 was provided by Gruppo LePetit, Milan, Italy.

Preparation of ATP-Sepharose. ATP was covalently linked to Sepharose-4B as described previously [4, 5]. In this process, ATP was attached to Sepharose 4B through the hydroxyl groups of its ribose via a 6-carbon bridge of adipic acid dihydrazide. Our preparations contained 5 to 13 μmol of nucleotide per ml of packed Sepharose as determined by phosphate analysis [6]. Chromatography on ATP-Sepharose was performed as described previously [4]. Specific conditions for labeling or pretreating the receptor samples are described in the figure legends.

Preparation of progesterone receptor. Freshly excised oviducts from White Leghorn laying hens were frozen in liquid nitrogen and stored at $-70°$ until use with little or no loss of hormone binding activity. The tissue was rinsed with cold 0.9% saline and homogenized with a Waring blender and then a "tissumizer" (Tekmar Model SDT) in three vol. (w/v) of buffer (10 mM Tris–HCl, 12 mM thioglycerol, 1 mM EDTA, 10% glycerol, 0.01 M KCl, pH 8). The homogenate was first centrifuged at 12,000 g for 10 min and then at 100,000 g for 90 min to obtain the cytosol fraction. In some cases, the cytosol was fractionated by ammonium sulfate precipitation as described previously [4] and the precipitates were stored at $-70°$.

White Leghorn chicks were primed with diethylstilbestrol for two weeks as described previously [7]. Cytosol was prepared from freshly removed oviducts as described above, but with two vol. of buffer containing either 40 mM Tris–HCl, 12 mM thioglycerol, pH 7.5 or 40 mM Tris–HCl, 12 mM thioglycerol, 10% glycerol, pH 8 (as indicated in figure legends).

Receptor activation and binding to ATP-Sepharose. In these experiments, elevated temperature (23°) or high salt conditions were employed as a means of receptor activation. For incubations at elevated temperatures, aliquots of chick oviduct cytosol were preincubated at 4° for 2 h with 20 nM [^{3}H]-progesterone and then incubated at room temperature for varying periods of time. The samples were then cooled on ice and tested for the binding of receptor to ATP-Sepharose (1 ml columns). For incubation in high salt,

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aliquots of cytosol containing 20 nM [^{3}H]-progesterone were incubated at 4° with 0.01 to 0.5 M KCl. Following a 1 h incubation, each sample was diluted 1:1 with buffer (40 mM Tris–HCl, 12 mM thioglycerol, 10 mM KCl, pH 8) before determining the binding of receptor to ATP-Sepharose.

Preincubation and nuclear-binding assay. Each experiment consisted of two main parts: preincubation of the [^{3}H]-progesterone-receptor sample, followed by a nuclear-binding assay. In the preincubation, 0.4 ml of cytosol labeled with 20 nM [^{3}H]-progesterone, was incubated at 23° or 0° with 0.15 ml 42% glycerol and 0.1 ml additive (inhibitor). For the nuclear-binding assay, the preincubation solutions were all cooled to 0° and incubated for one additional hour with 0.2 ml buffer (40 mM Tris–HCl, 12 mM thioglycerol, pH 7.5) and 0.15 ml purified oviduct nuclei containing 50–100 μg DNA. The nuclei had been prepared and stored according to Method II of Spelsberg *et al.*[8] (omitting the use of Triton X-100), and they were suspended in a buffer of 0.01 M Tris, 25% glycerol and 0.002 M $MgCl_2$, pH 7.5. At the end of the nuclear incubation, 3.5 ml wash (0.01 M Tris, 10% glycerol, 0.001 M $MgCl_2$ and 0.1% Triton X-100 buffer, pH 7.5) was added to each tube and the nuclei sedimented at 1000 *g* for 5 min. Supernatants were aspirated off, the nuclei resuspended in 3.5 ml wash and again recovered by centrifugation. Each nuclear pellet was then suspended in 0.5 ml H_2O and transferred to a scintillation vial. Ten ml of scintillation fluid consisting of Beckman Bio-Solv BBS-3, Spectrofluor PPO-POPOP and toluene, 2:1:16 (by vol.) were added. Radioactivity was determined with 40% efficiency. To measure nuclear binding, experimental points were determined from duplicate or triplicate incubations, and additional replicate incubations were carried out to assess the recovery of DNA. DNA was determined by the method of Burton[9].

Other methods. Unless otherwise indicated, radioactivity was determined by combining aqueous samples with 5 ml of a cocktail consisting of toluene (Baker), Triton X-100 (RPI), and spectrofluor (Amersham Searle), 100:521:42 by vol. The counting efficiency for tritium was 33% in a Beckman LS-250 Liquid Scintillation counter. Protein concentration was determined by the method of Lowry *et al.*[10], using BSA as the standard.

RESULTS

Binding of receptor to ATP-Sepharose

In our earlier report, the binding of progesterone receptor to ATP-Sepharose was demonstrated using receptor preparations that were first fractionated by ammonium sulfate precipitation to remove endogenous nucleotides [4]. These receptor preparations could be readily bound to the affinity resin and then eluted either with buffer containing ATP or with a high-salt buffer (e.g., 1 M KCl). The elution of progesterone receptor from ATP-Sepharose using an ATP gradient (0 to 0.1 M ATP) is illustrated in Fig. 1. With this procedure, essentially all of the adsorbed receptor was eluted in a single peak of material. Previous studies have shown that the first peak of non-adsorbed radioactivity represents unbound hormone. At the peak of gradient elution, the buffer concentration of ATP was approximately 30 mM, while the concentration of ATP linked to the column was about 13 μmol per ml of packed Sepharose. Additional studies have shown that the radioactivity that is adsorbed to ATP-Sepharose represents intact progesterone–receptor complex [4, 11]. It sediments as a 4S unit upon sucrose gradient centrifugation and its mobility during polyacrylamide gel electrophoresis is comparable to that of the original cytosol receptor.

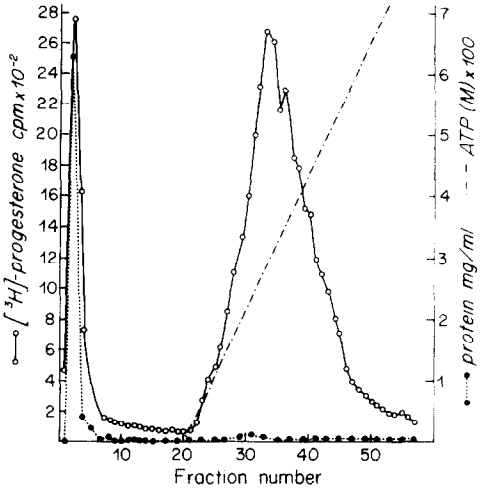

Fig. 1. ATP gradient elution of progesterone receptor from ATP-Sepharose column (5 ml) which was washed with buffer A (10 mM Tris–HCl, 12 mM monothioglycerol, 1 mM EDTA, and 20% glycerol, pH 8) containing 0.15 M KCl and fifteen 4.8 ml fractions were collected. The receptor complex was eluted with an ATP-gradient (0 to 0.1 M) prepared in buffer A plus 0.15 M KCl and 1.8 ml fractions were collected. Radioactivity (○——○) and protein (●——●) were measured in 0.1 ml portions of each fraction. ATP concentration (- - - -) of the gradient fractions was measured by diluting aliquots 1000-fold in water and reading optical density at 260 n.

Affinity chromatography on ATP-Sepharose is quite selective as indicated by the protein profile in Fig. 1. Less than five per cent of the total protein is adsorbed to the column whereas 80–100% of the progesterone receptor can generally be adsorbed and subsequently recovered by this procedure. It therefore offers a very effective means of receptor purification.

Analysis of hormone requirements. Since ATP binding may be of functional significance to hormone action, it was of importance to determine whether the presence of progesterone influenced this interaction. To test this, two receptor samples, identical except for the presence of [^{3}H]-progesterone in one, were chromatographed on ATP-Sepharose columns (Fig. 2). Following chromatography of the sample without hormone, [^{3}H]-progesterone was added to

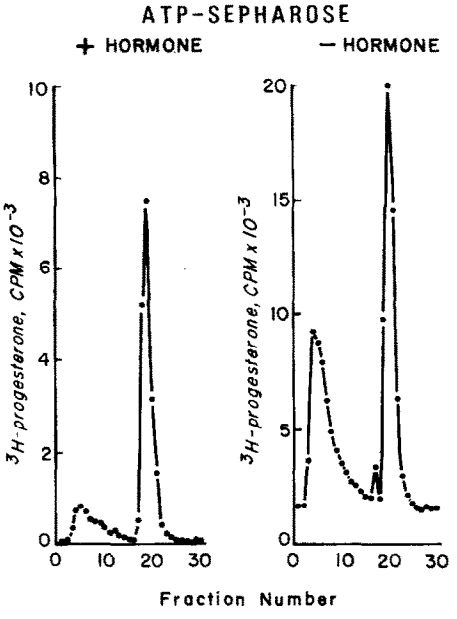

Fig. 2. ATP-affinity chromatography of progesterone receptor in the presence or absence of hormone. Identical receptor samples (1 ml) were incubated for 3 h at 4°C with and without 6 mM [^{3}H]-progesterone. The samples were then chromatographed on 1 ml columns of ATP-Sepharose. The columns were washed with buffer A plus 0.01 M KCl and then with buffer A plus 1 M KCl. Fifteen 0.7 ml fractions were collected in each case. Radioactivity was measured in 0.2 ml aliquots from each fraction obtained from the sample that was preincubated with [^{3}H]-progesterone (Left pannel). Aliquots (0.2 ml) from the fractions obtained after chromatography in the absence of hormone were incubated with 12 nM [^{3}H]-progesterone for 3 h at 4°C and the extent of [^{3}H]-progesterone binding was measured by charcoal adsorption assays (Right panel).

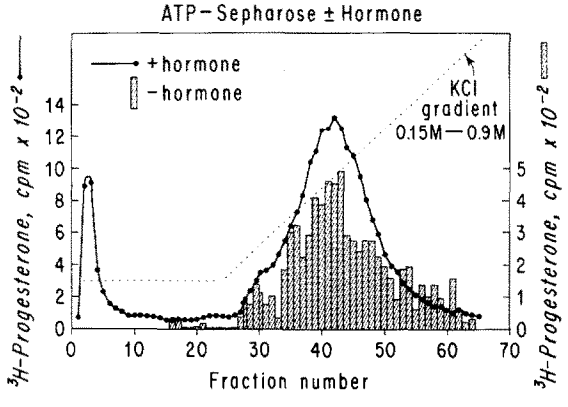

Fig. 3. ATP-affinity chromatography of progesterone receptor in the presence or absence of hormone. Two 2 ml receptor samples (without hormone or with 1 nM [^{3}H]-progesterone) were prepared as in Fig. 2 and were applied to identical 5 ml columns of ATP-Sepharose. The columns were first washed with buffer A plus 0.15 M KCl and 4.8 ml fractions were collected. The receptor was then eluted using a linear KCl gradient (0.15 to 0.9 M) in buffer A and 1.8 ml fractions were collected. [^{3}H]-Progesterone was measured in 0.1 ml portions of each fraction from the column which received hormone-receptor complex (●——●). With the column that received hormone-free receptor, hormone binding was subsequently monitored by charcoal adsorption assay as indicated by the histogram.

the column fractions which were then analyzed for hormone binding. The sample containing [^{3}H]-progesterone was applied to the column and binding was detected by a direct measurement of radioactivity in eluted fractions. In both cases, a major portion of the receptor was adsorbed to the column, leading to the conclusion that the receptor will bind to ATP-Sepharose even in the absence of hormone.

When the receptor samples prepared in the presence or absence of [^{3}H]-progesterone were chromatographed on ATP-Sepharose columns and eluted with a KCl gradient, the salt concentration needed for receptor elution was identical in both cases (Fig. 3). The relatively high salt concentration required for elution (0.5–0.6 M KCl) suggests that the ATP binding is of a rather high affinity and this affinity is apparently not altered significantly by the presence of progesterone.

Requirements for receptor activation. In contrast to the above results with salt-fractionated receptor, the receptor complex in freshly prepared cytosol had little or no affinity for ATP-Sepharose. This observation suggested that the receptor may first require an activation step similar to the activation process that is needed to bind the progesterone–receptor complex to isolated nuclei [12]. To test this hypothesis, aliquots of cytosol labelled with [^{3}H]-progesterone were incubated at room temperature for varying periods of time as a means of receptor activation. The samples were then chilled and applied to columns of ATP-Sepharose. The binding of receptor to ATP-Sepharose

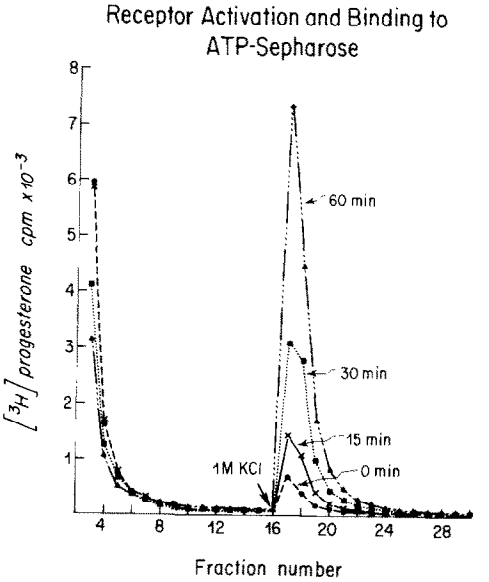

Fig. 4. The effect of room temperature activation on the binding of progesterone receptor to ATP-Sepharose. Chick oviduct cytosol was prepared in buffer containing 40 mM Tris–HCl, 12 mM thioglycerol, 10% glycerol, pH 8. Aliquots (0.5 ml) of cytosol were pre-incubated at 4°C for 2 h with 20 nM [^{3}H]-progesterone and then incubated at room temperature for the periods indicated. The amount of ATP binding by the 60 min sample represents 42% of the total progesterone receptor applied to the column.

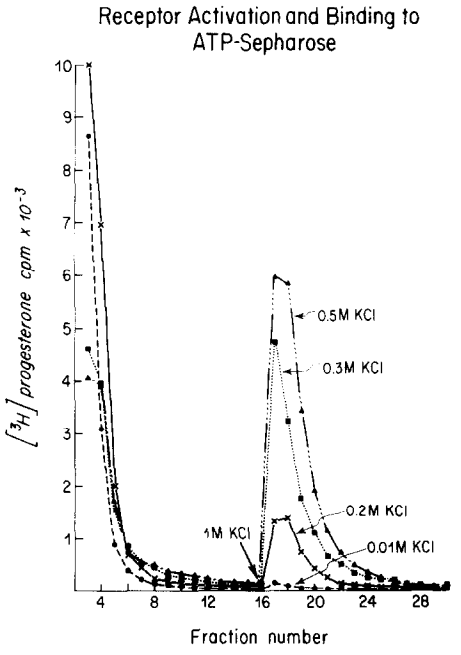

Fig. 5. The effect of salt activation on the binding of progesterone receptor to ATP-Sepharose. Aliquots (1.5 ml) of chick cytosol were incubated at 4°C in buffer containing 20 nM [^{3}H]-progesterone and the indicated concentrations of KCl. After a 1 h incubation, each sample was diluted 1:1 with buffer before determining binding to ATP-Sepharose. The amount of ATP binding by the 0.5 M KCl sample represents about 100% of the total progesterone receptor.

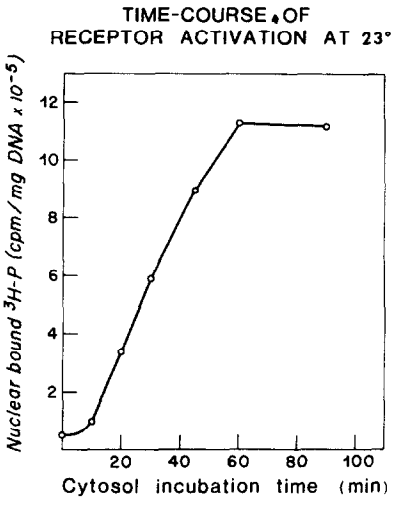

Fig. 6. Formation of activated [^{3}H]-progesterone-receptor in cytosol incubated at 23°C, showing time-dependence of the process. Preincubation of cytosol at 23°C and assay for nuclear binding at 0°C were carried out as described in Experimental. Reprinted from reference 14 by courtesy of Academic Press.

was greatly enhanced by pre-incubation of receptor at room temperature as illustrated in Fig. 4. With this procedure (temperature activation), the extent of receptor binding to ATP-Sepharose was found to be as high as 60% of the total cytosol receptor (determined by charcoal adsorption [7]. Studies on the time course of incubation showed a close relationship between the capacities of receptor to bind both ATP-Sepharose and isolated nuclei (see Fig. 6). Therefore, the same activation processes may be required for both binding activities.

Receptor activation can also be accomplished by a brief exposure to high salt conditions [13]. This treatment also enhanced the binding of receptor to ATP-Sepharose as illustrated in Fig. 5. Maximum binding was observed after treating the cytosol receptor with 0.5 M KCl followed by dilution to 0.25 M KCl. Under these conditions, 80–100% of the progesterone–receptor complex acquires the ability to bind ATP-Sepharose. Since this procedure avoids temperature elevation, it can be carefully controlled and can be used to activate almost all of the receptor complex.

Inhibition of the receptor-nucleotide interaction. A recent report from this laboratory described the use of two inhibitors which block binding of the [^{3}H]-progesterone–receptor complex to isolated nuclei [14]. In these experiments, chick oviduct cytosol containing [^{3}H]-progesterone was heated at 23° to convert the hormone–receptor complex to the activated form which can bind to oviduct nuclei at 0°. The time-course of this activation, as measured by nuclear binding, is illustrated in Fig. 6. When either of the two inhibitors, o-phenanthroline or rifamycin AF/013 were preincubated with the cytosol, the nuclear binding of the progesterone–receptor complex was markedly inhibited (Fig. 7). o-Phenanthroline is an organic metal chelator and its effectiveness toward progesterone and estrogen receptor suggests that these receptors may be metalloproteins [14, 15]. The other inhibitor, rifamycin AF/013, is an antibiotic derivative that is best known for its ability to inhibit

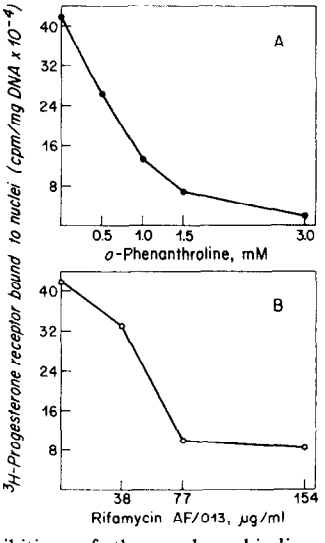

Fig. 7. Inhibition of the nuclear binding activity of [^{3}H]-progesterone receptor in oviduct cytosol when o-phenanthroline or rifamycin AF/013 are preincubated with the cytosol for 45 min at 23°C. Reprinted from reference 14 by courtesy of Academic Press.

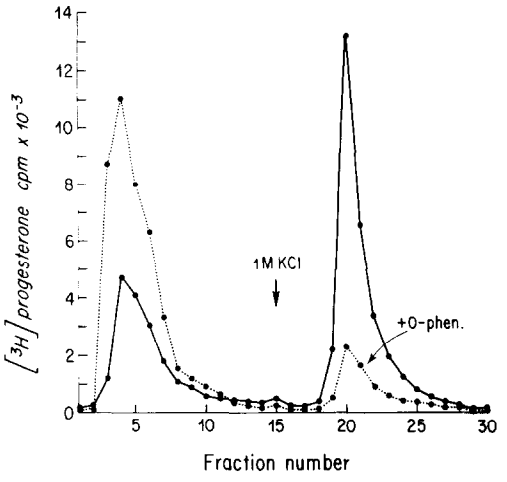

Fig. 8. Effect of o-phenanthroline on the binding of progesterone receptor to ATP-Sepharose. Ammonium sulfate-precipitated receptor from hen oviduct was dissolved in buffer (10 mM Tris–HCl, 12 mM thioglycerol, 20% glycerol, pH 8.0) and preparations containing 200 μl of receptor, 12 nM [^{3}H]-progesterone and 5 mM o-phenanthroline were incubated at room temperature for 30 min. The final concentration of glycerol in the sample was 15% and vol. was made up to 0.5 ml with the above buffer. Following this, the samples were brought to 4°C for 2 h before layering on 1 ml ATP-Sepharose columns. The columns were washed first with buffer containing 0.01 M KCl and then with 1 M KCl. Fifteen fractions (0.6 ml/fraction) were collected in each case at a flow rate of 2 ml/min. A sample without inhibitor served as the control.

DNA and RNA polymerases [16, 17]. Further studies show that these inhibitors do not simply block the receptor activation process since they are also effective when added following receptor activation [14].

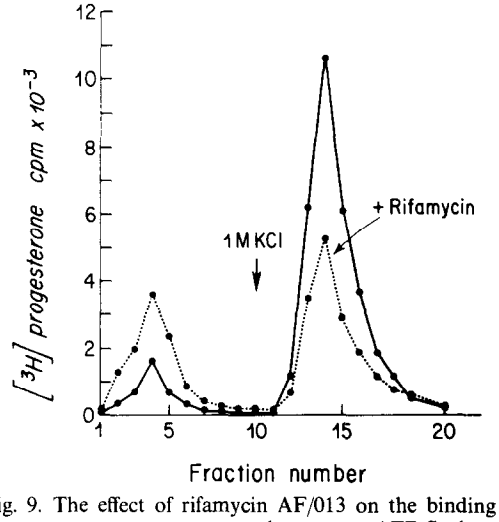

Fig. 9. The effect of rifamycin AF/013 on the binding of progesterone–receptor complex to ATP-Sepharose. Ammonium sulfate-fractionated receptor was dissolved and labelled with [^{3}H]-progesterone as described in Fig. 7. The receptor complex was treated with rifamycin AF/013 (100 μg/ml) for 2 h at 4°C. Sample with no inhibitor served as control. The chromatography procedure was similar to that described in Fig. 8.

Since these inhibitors block nuclear binding of the progesterone receptor, it was of interest to see if they would also interfere with binding of the receptor to ATP-Sepharose. Figures 8 and 9 illustrate that this is the case. Both o-phenanthroline and rifamycin AF/013 reduce the binding of progesterone receptor to ATP-Sepharose. In these experiments, the inhibitors were used at a concentration that would demonstrate the inhibition of ATP binding without altering the binding of steroid to receptor. More complete inhibition can be achieved at higher concentrations, but these concentrations also reduce the binding of progesterone to receptor [18].

DISCUSSION

While the avian progesterone receptor is capable of selectively binding ATP, the biological role of this interaction remains unclear. The specificity of the interaction [4] suggests that the receptor contains a unique binding site for ATP. However, it is also possible that ATP binding could be related to the known interaction of steroid receptors with phosphate (phosphocellulose) and with polynucleotides such as DNA. While some relationship among these interactions may exist, there are also clear differences. Elution of the progesterone receptor as a single peak from ATP-Sepharose columns (Figs. 1 and 3) would suggest homogeneity in relation to ATP binding. However, by other criteria, the receptor is known to be composed of two similar hormone-binding components (A and B) that are distinguishable by ion exchange chromatography [19, 20]. The two components appear to differ slightly in size and in their ability to bind to DNA and chromatin [3, 21]. The present results would indicate, however, that the two forms bind ATP in a similar manner. On the other hand, chromatography on phosphocellulose distinguishes between the A and B receptor components and this interaction is disrupted by salt elution conditions that are below the effective ionic strength required to disrupt ATP binding. The binding of receptor to DNA is also more sensitive to ionic strength, and this interaction appears to be a property of only receptor component A [21].

Our earlier studies [4] were carried out using receptor that had been subjected to ammonium sulfate precipitation. This preparation has been shown to be in an "activated state" in the sense that it has the ability to bind to oviduct nuclei in a cell-free system without temperature elevation [12]. The receptor in the original cytosol fraction can also be activated for nuclear uptake by a process which requires the hormone plus an incubation period at elevated temperature (e.g., 30 to 60 min at 23°) [12]. The present results show that activation of receptor (by elevated temperature or high salt treatment) is required for the binding of receptor to ATP. Non-activated receptor has little or no affinity for ATP-Sepharose. This observation may be related to some other recent

studies from this laboratory which show that the addition of various nucleotides can either inhibit or accelerate the progesterone receptor activation process, depending upon the concentration of nucleotide used [22]. However, additional studies are needed to establish the possible involvement of nucleotides in receptor activation.

Since ATP is bound more readily by activated receptor, this interaction may be involved in a function of the receptor at sites on the nuclear chromatin. This possibility is supported by the studies with o-phenanthroline and rifamycin AF/013. These inhibitors block binding of the hormone–receptor complex to both nuclei and to ATP-Sepharose. These results suggest that nucleotide binding sites on the receptor may be closely associated with a region that participates in the nuclear binding process. However, it is also possible that two distinct sites of interaction exist which are both altered as a result of conformational changes in the receptor caused by the inhibitors. We have recently identified another compound, aurintricarboxylic acid (a triphenylmethan dye) which blocks the binding of receptor to nuclei and to ATP-Sepharose [18]. This compound is a known inhibitor of some nucleotide binding proteins such as RNA polymerase [23], QB replicase [23] and certain ribosomal proteins [24]. These inhibitors should prove to be useful probes for additional characterization of nucleotide binding sites on the receptor molecule.

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DISCUSSION

Jensen. This is a very nice demonstration of the interaction with ATP and the need for activation. I am only concerned about the known ability of the activated form of receptors, at least estrogen and glucocorticoid receptors, to bind not only to DNA and chromatin but to polyanions in general, such as phosphocellulose as shown by Baulieu. Do you think you are observing a specific binding to a nucleotide type of phosphate or could this be a non-specific binding to what might be considered to be a polyphosphorylated column?

Toft. We think that it's a specific interaction but our data is limited at the moment. It is very possible that the same region of the receptor molecule binds the phosphate of phosphocellulose and ATP-Sepharose. The affinities are quite different in that one needs about 0.2 to 0.3 M potassium chloride to elute receptor from phosphocellulose where 0.5 to 0.6 M potassium chloride is needed to elute from ATP-Sepharose. Furthermore, the competition studies which we have published earlier demonstrate some preference for ATP as opposed to other nucleotides. We have also tried an ATP-Sepharose resin where the ATP was attached to the resin through the base rather than the sugar. While this resin binds receptor, it is not as effective as the resin that we are now using.

Jungblut. When I read the summary of your paper, it struck me that we might have some rather old supporting evidence. The analysis of the first receptor preparations isolated by specific adsorption showed the presence of aminoacids, solvent-extractable and non-extractable phosphorous and the material gave a positive orcinol reaction. It was adsorbed from extracts, which from our present knowledge could have contained activated receptors (Jungblut P. W., Hätzel I., Desombre E. R. and Jensen E. V., in *Wirkungsmechanismen der Hormone*, Springer-Verlag, Berlin 1967).

Birmingham. I wanted to ask whether cyclic nucleotides do anything in your system.

Toft. We have not seen any effect of cyclic nucleotide so far but the main thing we have tried is the affinity column procedure in that cyclic AMP or cyclic GMP were tested as competitors for binding to ATP-Sepharose. They are not competitors. Also, if we make affinity resins with these cyclic nucleotides, they are not nearly as effective as the ATP-Sepharose.

Liao. Have you tried to attach ADP or AMP to Sepharose and compares their effectiveness?

Toft. We have not tried them attached to the Sepharose. We have simply tried them as competitors to the binding of receptor to ATP-Sepharose.

Notides. If the progesterone receptor is binding two ligands, the binding sites may be linked. Have you looked to see whether ATP alters the progesterone-binding kinetics of the receptor?

Toft. Yes, we have and we have been able to find no evidence for this in the chick oviduct system. However, that was actually one of our earlier ideas. In getting into this, we were originally working with progesterone receptor from the cow uterus and in that system we were looking at several different compounds to see if they would stabilize the progesterone receptor complex. We found that the addition of ATP would stabilize or would enhance the association of the progesterone to the receptor somewhat. However, this was a small effect and difficult to study. Therefore, we went to the affinity column procedure to demonstrate the interaction more clearly. Since the chick oviduct receptor is a little easier to handle than that of the cow uterus, we went to that system. But unfortunately in the chick oviduct system, we see no effect of added ATP on the binding of steroid, and at least with partially purified receptor, we see no effect of the hormone on binding to ATP.

Notides. Your observation is interesting, since I would imagine the changes you should expect to see may be very subtle, such as the 10% difference you noted.

Toft. Right, that may be and the mammalian progesterone receptor is much more amenable to these kinds of experiments because it is so sensitive to various environmental conditions. Glycerol, for example, has a big effect on binding to the mammalian progesterone receptor but a minor effect on chick oviduct receptor.

Munck. Do you know whether the normal receptor has ATP attached?

Toft. No we do not. That would be very difficult to determine, I think, because what are you going to call normal receptor. In the initial cytosol, the endogenous ATP concentration is somewhere around 1 millimolar and how do you tell if any of this is bound to such a minor component as the receptor?

Munck. I recall a report, I think it was from either Dr. Jensen's or Dr. O'Malley's lab, in which they found no phosphorous in the receptor.

O'Malley. In the pure receptor we found no detectable phosphate.

Munck. And this is a receptor which can do everything you expect it to do?

O'Malley. Yes.

Jensen. We do have two types of evidence that suggest that the estrogen receptor protein may contain phosphorus, but this is not established with certainty.

Munck. What is the stoichiometry of ATP binding sites to hormone binding sites?

Toft. We really have no idea. What we want to do at the moment is obtain highly purified receptor and study the stoichiometry of binding. Also we would like to study the possibility that either ATP binding modifies receptor structure or that the receptor possibly metabolizes or breaks down the ATP.