ATP–PP$_i$ exchange activity of progesterone receptor

(avian oviduct/receptor purification/affinity chromatography/gel electrophoresis)

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ABSTRACT   Progesterone receptor preparations from avian oviduct catalyze a pyrophosphate (PP$_i$)–exchange reaction between ATP and ^{32}P-labeled PP$_i$. The reaction requires ATP exclusively and is Mn^{++}-dependent. This enzyme activity is detectable in receptor preparations that have been purified extensively by chromatography on ATP-Sepharose and DEAE-Sephadex columns. Polyacrylamide gel electrophoresis of purified preparations reveals a comigration of [^{3}H]progesterone–receptor complex and the enzyme activity. The PP$_i$-exchange reaction is inhibited by both o-phenanthroline and rifamycin AF/013, which also block the nuclear binding of progesterone receptor. These findings indicate that progesterone receptor may be an enzyme or a subunit of an enzyme that is active in nucleotide metabolism.

A recent report from this laboratory identified an interaction between the progesterone receptor and ATP (1). This interaction was demonstrated 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. These results suggested that ATP may play a role in some aspect of receptor function.

Efforts were made to follow the fate of receptor-bound nucleotide and to determine whether or not ATP retained its integrity after it was bound to the receptor. In this report, highly purified receptor preparations are shown to have enzymatic activity for ATP–pyrophosphate exchange. That this activity is a property of the receptor protein is indicated by its copurification, its electrophoretic mobility in acrylamide gels, and its sensitivity to two inhibitors (2) that block binding of [^{3}H]progesterone–receptor complex to isolated nuclei.

MATERIALS AND METHODS

All reagents were of analytic grade and were made up in glass-distilled water. Nucleotides were from Schwarz/Mann; Sepharose 4B, DEAE-Sephadex-A-25, and Dextran T-70 from Pharmacia, Uppsala, Sweden; carboxymethyl-cellulose from Whatman Biochemical, Ltd; acrylamide and N,N'-methylenebisacrylamide from Bio-Rad; o-phenanthroline from Fisher Scientific; dithiothreitol from Calbiochem; thioglycerol, activated charcoal, unlabeled progesterone, bovine serum albumin, and Coomassie blue from Sigma; and [1,2-^{3}H]progesterone (50 Ci/mmol) and Na$_4$^{32}P$_2$O$_7$ (1.005 Ci/mmol) were from New England Nuclear. Rifamycin AF/013 was provided by Gruppo Lepetit, Milan, Italy.

Buffer A contained 10 mM Tris·HCl, 12 mM monothioglycerol, 1 mM EDTA, and 20% (vol/vol) glycerol, pH 8.0 (supplemented with KCl as indicated). Buffer A without EDTA was used to study the effect of inhibitors on PP$_i$-exchange.

Buffer B contained 10 mM Tris·HCl and 1 mM EDTA, pH 8.0.

Buffer C contained 0.3 M K$_2$H PO$_4$, 20% glycerol, and 12 mM thioglycerol, pH 8.0.

Preparation of ATP-Sepharose. ATP was covalently linked to Sepharose-4B as described (1). Our preparations contained 5–13 μmol of nucleotide per milliliter of packed Sepharose, as determined by phosphate analysis (3).

Preparation of DEAE-Sephadex and Carboxymethyl-Cellulose. The ion exchange resins were precycled, degassed, and equilibrated in buffer B as recommended by the manufacturer. Before use, the resins were equilibrated with buffer A plus 0.01 M KCl and packed into columns.

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 cytosol was prepared after homogenization in buffer A plus 10% glycerol and 0.01 M KCl, pH 8.0, as described (1).

White Leghorn chicks were primed with diethylstilbestrol for 2 weeks as described (4). Cytosol was prepared from freshly removed oviducts as described above, but in buffer without glycerol.

Purification of Progesterone Receptor. (A) *ATP-Sepharose-I.* After ammonium sulfate fractionation, the progesterone receptor has a high affinity for ATP-Sepharose (1). However, recent studies have shown that the cytosol progesterone receptor acquires the ability to bind ATP-Sepharose only after it is "activated" either by incubation at elevated temperature (e.g., 23° for 30 min) or by exposure to high ionic conditions (as occurs during salt precipitation) (5). Therefore, the receptor protein in the initial cytosol preparation has little or no affinity for ATP-Sepharose; this was used to some advantage in receptor purification. The fresh cytosol was treated with ATP-Sepharose to remove other ATP binding proteins and increase the effectiveness of the second ATP-Sepharose step (see below). The cytosol (400–500 ml) was passed through a 20-ml column of ATP-Sepharose at 4° with a flow rate of about 2 ml/min. The column was washed with 20 ml of buffer A + 0.01 M KCl, and the entire flowthrough was used for the next step. This preparation contained about 80% of the cytosol protein and usually greater than 80% of the receptor. The progesterone binding activity (pmol/mg of protein) was usually equivalent to that of the original cytosol. Cytosol from chick oviducts was labeled with [^{3}H]progesterone at this point by incubating in ice for 2 hr with 2 × 10^{-8} M [^{3}H]progesterone (diluted to specific activity 5 Ci/mmol) and 6 × 10^{-7} M cortisol (to saturate corticoid binding globulin sites). Hen oviduct preparations were not labeled until step C.

(B) *Ammonium sulfate precipitation.* This step was as described (1) except that the final ammonium sulfate concentration was increased from 35 to 45% of saturation.

(C) *ATP-Sepharose-II.* The precipitated receptor was redissolved in one-tenth the original cytosol volume of buffer A + 0.01 M KCl and was centrifuged to remove undissolved material. At this point, hen cytosol was incubated for 2 hr at 4° with 2 × 10^{-7} M [^{3}H]progesterone (diluted to specific activity 5 Ci/mmol). The sample was then applied to a 5-ml column of ATP-Sepharose at a rate of 2 ml/min. The column was then

washed sequentially with 30 ml of buffer A + 0.2 M KCl, 30 ml of buffer C, and then 30 ml of buffer A + 0.2 M KCl. Finally, the receptor was eluted in about 20 ml of buffer A + 1 M KCl. Pooled fractions containing receptor were dialyzed for 2 hr in 1 liter of buffer A + 0.01 M KCl with two buffer changes.

(D) *Carboxymethyl-cellulose.* After dialysis, the preparation was passed through a 10-ml column of carboxymethyl-cellulose at a rate of 2 ml/min. The receptor, in the flowthrough fractions, was immediately applied to DEAE-Sephadex (step E). The carboxymethyl-cellulose step caused little apparent purification, but often increased the effectiveness of step E.

(E) *DEAE-Sephadex.* The preparation from step D was applied to a 25-ml column of DEAE-Sephadex at a rate of 2 ml/min. The column was washed with 50 ml of buffer A + 0.01 M KCl and with 50 ml of buffer A + 0.15 M KCl. Receptor form "A" (6–8) was removed with this step but was not used in the present study. The "B" form of the progesterone receptor was then eluted using an 80-ml linear gradient of 0.15–0.5 M KCl in buffer A. The peak fractions of radioactivity were pooled and mixed with an equal volume of saturated ammonium sulfate for 2–4 hr at 4°. The precipitate was not visible but could be recovered by centrifugation for 30 min at 53,000 × g. It was either used immediately or was stored at −70° for 1–3 days without significant loss of binding or enzyme activity.

Hormone Binding Assay. At each step of receptor purification, the [³H]progesterone binding activity was measured as follows: hormone binding was measured in the cytosol preparations before and after ATP-Sepharose treatment by use of the charcoal adsorption method (4, 9). Binding was determined at five hormone concentrations and was expressed as a Scatchard plot (10). A high affinity binding was observed in all cases, and the total concentration of binding sites was determined from the x-intercept. After ammonium sulfate fractionation of hen oviduct receptor, binding was determined by the charcoal method using one saturating concentration of [³H]progesterone $(2 \times 10^{-7}$ M). In this case, background determinations were made using aliquots of receptor that were denatured by incubation at 37° for 2 hr. In addition, 10^{-6} M unlabeled progesterone was added to these samples. Since a saturating level of [³H]progesterone was added to the cytosol from chick oviducts, additional hormone was not added to the ammonium sulfate fractions. In this case, the amount of [³H]progesterone that precipitated during ammonium sulfate fractionations was assumed to represent the total binding sites.

The quantity of [³H]progesterone that migrated with the adsorbed protein on column chromatography was assumed to represent the total receptor binding activity, and additional tests for binding were not performed.

Assay for ATP-PP$_i$ Exchange Activity of Progesterone Receptor. An assay used for measuring the exchange of pyrophosphate between ATP and ³²PP$_i$ was developed by modifying the method of Cranston *et al.* (11). The incubation mixture (0.5 ml) contained 10 mM Tris·HCl, 2 mM dithiothreitol, 10 μg of bovine serum albumin, 4 mM manganese chloride, 120 nM Na$_4$³²P$_2$O$_7$, and 1 mM nucleotide. Duplicate aliquots containing excess (100 μM) unlabeled PP$_i$ were used for background determination. After incubation at 35° for 30 min the reaction was terminated by chilling in ice and adding 0.4 ml of solution containing 0.1 M sodium pyrophosphate and 1% bovine serum albumin. After this, 0.2 ml of 10% charcoal in buffer A (containing 1% dextran, but no glycerol) was added to each assay tube. Five percent trichloroacetic acid (5 ml) was added to the above mixtures, which were then kept in ice for 5 min. The contents of the assay tubes were poured over 2.4-cm glass fiber filters (Reeve Angel) to remove nucleotide adsorbed to charcoal.

The filters were washed with 50 ml of 1% trichloroacetic acid, and the radioactivity was determined by liquid scintillation.

Effects of Rifamycin AF/013 and o-Phenanthroline. Purified receptor preparations were pre-incubated at room temperature for 40 min with various concentrations of rifamycin AF/013 or o-phenanthroline in the presence of buffer containing 10 mM Tris·HCl, 12 mM thioglycerol, and 10% glycerol. After preincubation, the samples were assayed for PP$_i$ exchange as described above. Stock solutions of the inhibitors were prepared in dimethylsulfoxide so that the final concentration of solvent in the assay mixture never exceeded 5%.

Polyacrylamide Gel Electrophoresis. The methods used were as described by Miller *et al.* (12) except that the gel composition was 5% total acrylamide with 3% crosslinking (bisacrylamide comprised 3% of the total acrylamide). The gels also contained 20% glycerol and were run in a Tris-glycine buffer system (12). The purified receptor was redissolved from ammonium sulfate precipitates in buffer A and was dialyzed for 1 hr in this buffer. Fifty microliters of 30% glycerol + 0.1 M mercaptoacetic acid and 0.1 M Tris·HCl, pH 8.0, were layered on each gel, followed by 50–100 μl of receptor. Electrophoresis was carried out for 1 hr at 1 mA per gel and then for 3–4 hr at 2 mA per gel, until the marker dye (methylene blue) had reached the end of the gel. One gel was stained overnight with 0.1% Coomassie blue in 7% acetic acid and then destained by washing with 7% acetic acid over 1–2 days. The second gel was sliced into 2-mm sections for determining the migration of [³H]progesterone–receptor complex. The sections were placed in counting vials containing 5 ml of toluene-based scintillation fluid as described below but without Triton X-100. The third gel was sliced as above, and each section was crushed and taken up in 200 μl of buffer A minus EDTA. Assays for ATP-PP$_i$ exchange activity were performed on each gel fraction for comparison with the migration of [³H]progesterone–receptor complex.

Other Methods. Radioactivity was determined as described (1). Protein concentration was determined by staining with Amidoschwarz as described by Schaffner and Weissmann (13), with bovine serum albumin as the standard.

RESULTS

In our earlier report (1), we demonstrated an interaction between ATP and progesterone receptor. This interaction was shown by ATP-Sepharose affinity chromatography, using crude receptor preparations that were fractionated by ammonium sulfate precipitation. In the present studies, receptor preparations from either hens or chicks were used after extensive purification (Table 1). The most effective step in purification was chromatography on ATP-Sepharose. When this was followed by DEAE-Sephadex chromatography the specific activity (bound progesterone per mg of protein) usually did not increase, but often decreased due to receptor instability. However, when preparations were analyzed by acrylamide gel electrophoresis, a number of protein bands were eliminated by DEAE-Sephadex chromatography, and this step was therefore included. Additional fractionation was unsuccessful due to receptor instability.

Our purified receptor preparations were found to catalyze an exchange reaction that incorporates ³²PP$_i$ into ATP. The basic requirements for this enzyme activity are shown in Fig. 1A. The reaction is totally dependent on the presence of ATP, Mn^{++}, and the receptor preparation. Other divalent cations (Ca^{++} or Mg^{++}) will not substitute for Mn^{++} in this reaction (data not shown). A 1000-fold excess of unlabeled PP$_i$ reduces ATP labeling to the background level (Fig. 1A), whereas inor-

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Table 1. Receptor purification

	Total protein (mg)	Total R (pmol)	SA* (pmol/ mg)	Yield (%)	Purifi- cation (times)
Hen					
Cytosol	31,300	7070	0.23	100	1
ATP-S-I	25,900	6340	0.24	90	1
AS ppt	394	1160	3.0	16	13
ATP-S-II	4.7	1110	234	16	1020
DEAE-S	0.3	129	391	2.0	1700
Chick					
Cytosol	3,840	3340	0.87	100	1
ATP-S-1	2,910	2700	0.93	81	1
AS ppt	240	2500	10	75	12
ATP-S-II	3.7	1450	396	43	455
DEAE-S†	1.4	306	219	9	250

ATP-S, ATP-Sepharose; AS ppt, ammonium sulfate precipitate; DEAE-S, DEAE-Sephadex.

* Theoretical maximum specific activity (SA) estimated to be 8696 pmol/mg of protein, assuming one binding site per receptor (R) of molecular weight 115,000.

† This step was preceded by carboxymethyl-cellulose treatment (see Materials and Methods).

ganic phosphate at the corresponding concentration has no effect (data no shown). This would indicate that PP_i and not P_i is released from ATP during this exchange reaction. Further analysis of the reaction shows an absolute specificity for ATP among the nucleotides tested (Fig. 1B). Other nucleotides, including 3':5'-cyclic AMP, deoxy-ATP, adenylimidodiphosphate
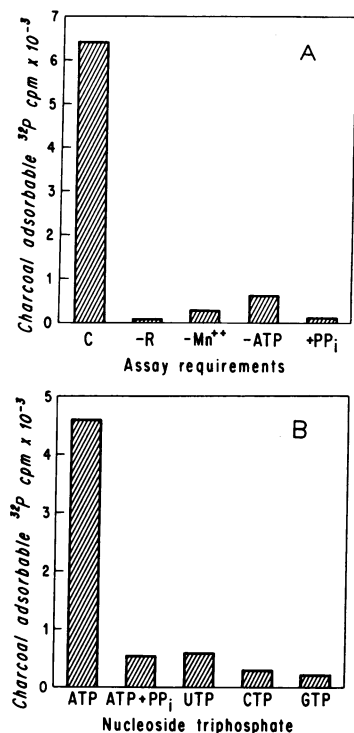

FIG. 1. Requirements for ATP-^{32}PP$_i$ exchange assay. Purified preparations of progesterone receptor (R) were incubated with 4 mM MnCl$_2$ (Mn^{++}), 1 mM ATP, and 120 nM Na$_4$^{32}P$_2$O$_7$ in a final assay volume of 0.5 ml. A 1000-fold excess of unlabeled PP$_i$ was used for background determination. As a measure of nucleotide specificity, other nucleoside triphosphates (1 mM) were tested for their ability to replace ATP as substrate in the PP$_i$-exchange assay (panel B).
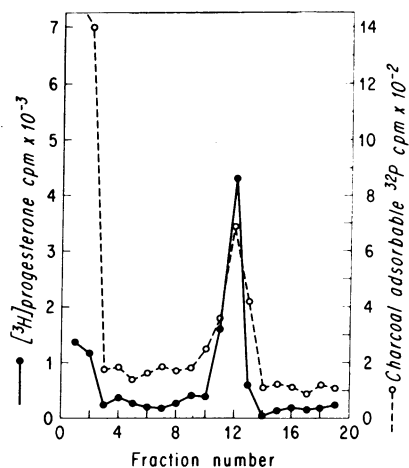

FIG. 2. Electrophoretic comigration of the [^{3}H]progesterone–receptor complex and PP$_i$-exchange activity. The receptor was purified from chick oviduct as illustrated in Table 1 and had a specific activity of 417 pmol/mg at step 4 and 290 pmol/mg at step 5. After electrophoresis, two gels were sectioned and analyzed for [^{3}H]progesterone and for enzyme activity. The migration was from left to right.

(an analogue of ATP), ADP, and AMP, failed to substitute for ATP (data not shown). Following the procedures of Cranston *et al.* (11), incorporation of ^{32}PP$_i$ into ATP was verified in our studies by thin-layer chromatography of the radioactivity adsorbed to charcoal. In addition, the radioactive ATP was not precipitated by the trichloroacetic acid used during the charcoal adsorption step and was, therefore, not bound in a macromolecular complex.

Although we were unable to increase the purity of our receptor preparations by additional column chromatography, these preparations could be analyzed very successfully by polyacrylamide gel electrophoresis. Fig. 2 illustrates the electrophoretic migration of the [^{3}H]progesterone–receptor complex on acrylamide gels. After electrophoresis, the gels were sliced into 2-mm sections which were analyzed for radioactivity or for enzyme activity. Greater than 50% of the hormone-receptor complex entered the gel and most of this migrated as a well-defined peak of radioactivity. When a parallel gel was analyzed for ^{32}PP$_i$-exchange activity, the migration of this activity was identical to that of the major receptor peak. This comigration of activities has been observed consistently in six separate receptor preparations. A third gel was stained for protein with Coomassie blue, and several well-resolved protein bands were apparent (Fig. 3). By comparison with gels containing ovalbumin standards, we estimate the visible band migrating with receptor to contain approximately 1 µg of
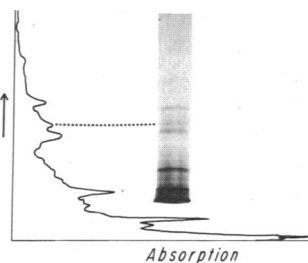

FIG. 3. Protein analysis by acrylamide gel electrophoresis. A third gel from the experiment illustrated in Fig. 2 was stained with Coomassie blue. The optical density profile was measured at 560 nm in a Beckman Acta C11 spectrophotometer. The position of the receptor is indicated by the broken line.

protein. The electrophoretic sample contained about 1.5 μg of receptor (estimated by bound progesterone), and it is therefore conceivable that the visible band represents receptor protein. From the optical density profile in Fig. 3, we estimate that about 5% of the protein migrates in the receptor-enzyme region. Therefore, the gel electrophoresis could possibly be equated with an additional 20-fold purification of the receptor. If this were so, the receptor could be calculated to be at least 50% pure (see Table 1, footnote*); however, the specific activity of material eluted from the gel could not be accurately determined.

As additional support for the identity of receptor with the enzyme activity, the effects of o-phenanthroline and rifamycin AF/013 on the PP$_i$-exchange activity were tested. o-Phenanthroline is an organic chelating agent, while rifamycin AF/013 is an antibiotic derivative. Both agents block binding of the progesterone-receptor complex to isolated nuclei (2). These inhibitors were also found to block the ^{32}PP$_i$-exchange activity (Fig. 4). In this experiment the effective concentration ranges for rifamycin AF/013 (50–250 μg/ml) and o-phenanthroline (1–5 mM) are very similar to the concentrations that were previously shown to block the binding of receptor to isolated nuclei (rifamycin AF/013, 50–100 μg/ml; o-phenanthroline, 0.5–1 mM) (2). While the inhibition by o-phenanthroline could possibly be due to chelation of Mn^{++} in the reaction mixture, this was found to be unlikely. Significant inhibition with o-phenanthroline was observed using a final concentration below that of Mn^{++}, and the addition of higher Mn^{++} concentrations to the assay did not counteract the inhibitory effect of the chelator (data not shown).

DISCUSSION

This report demonstrates that highly purified preparations of the avian progesterone receptor catalyze ATP-PP$_i$ exchange. ATP, which had previously been shown to bind to progesterone receptor (1), is apparently split into an AMP-enzyme complex and PP$_i$, and can be regenerated into a labeled form in the presence of ^{32}PP$_i$. This reaction is catalyzed by various enzymes of protein synthesis and nucleic acid metabolism, including DNA-dependent RNA polymerases (14–16), DNA polymerases (17–19), RNA ligase (11), DNA ligase (20), amino acid:tRNA ligases (21–23), and a polyadenylate polymerase (24). The metabolic significance of PP$_i$-exchange reactions is reviewed elsewhere (18, 25).

In studying the possible metabolism of ATP by the progesterone receptor, the PP$_i$-exchange assay was used because of its extreme sensitivity. An additional advantage of this assay in the detection of an unknown enzyme activity is that it involves a reversible, partial reaction that has a minimum of assay requirements. In the present study, the exchange reaction utilized ATP specifically and was dependent on Mn^{++} for its divalent cation requirement. Many of the nucleotide polymerases cited above require a polynucleotide template for optimal PP$_i$-exchange activity. However, preliminary results show that the addition of DNA or RNA to receptor preparations does not stimulate the exchange activity.

Our studies to date have been totally concerned with establishing an identity between this activity and the progesterone receptor. Therefore, details of the reaction kinetics and mechanism have not yet been analyzed and the exact reaction remains speculative.

The results of this study are not absolutely conclusive since the receptor preparations, while extensively purified, still contained a considerable amount of contaminating protein. A
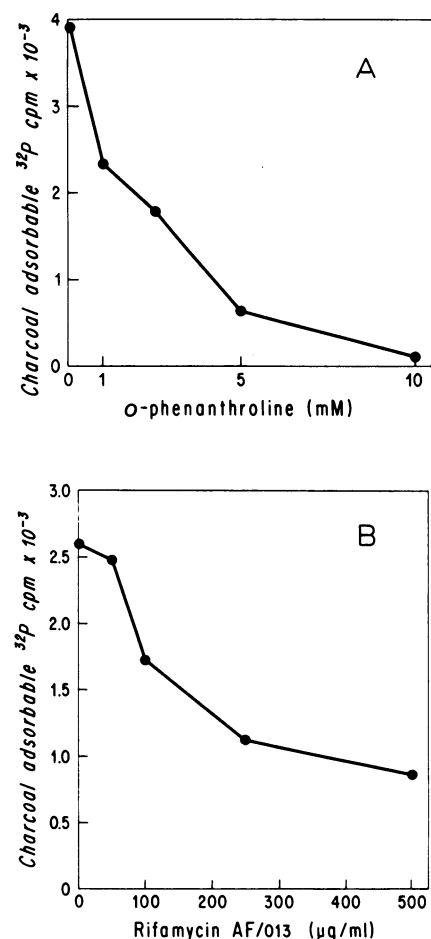

FIG. 4. Inhibition of the PP$_i$-exchange activity of purified progesterone receptor preparations by o-phenanthroline (A) and rifamycin AF/013 (B). Receptor preparations were first incubated at room temperature for 40 min with different concentrations of the inhibitors. After this incubation, the other ingredients of the PP$_i$-exchange assay were added and the samples were assayed as described in *Materials and Methods*. The inhibitor concentrations shown on the abscissa represent those in the first-incubation mixture. The samples were then diluted 2.5-fold for the exchange assay.

recent report by Kuhn *et al.* (7) demonstrated the purification of progesterone receptor using steroid affinity chromatography. With this technique they were able to purify the receptor to near homogeneity. However, similar efforts in our laboratory have not been very successful. Difficulty in purifying steroid receptors has been encountered by many laboratories. However, we believe that the present results are quite convincing. By using an approximate molecular weight of 115,000 for the receptor (7) and the specific activity (bound hormone per mg of protein), we estimate that the receptor represented about 2–5% of the total protein mass in our preparations from the hen and chick oviduct. However, this could be an underestimate for two reasons: (a) hen oviducts contain endogenous unlabeled progesterone for which correction was not made; and (b) since the recovery of receptor during purification was quite low, the final preparations could contain significant quantities of receptor that had lost the ability to bind hormone through partial denaturation. An additional purification of about 20-fold should have been accomplished by gel electrophoresis, thus removing most of contaminating protein. Attempts were made to separate receptor from the enzyme activity with other fractionation procedures, none of which were successful. These included: (a)

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phosphocellulose column chromatography with a KCl gradient elution, (b) ATP-Sepharose column chromatography with an elution by free ATP, (c) hydroxylapatite column chromatography with a step-wise phosphate elution, and (d) DEAE-cellulose column chromatography with a KCl gradient elution.

Two forms of progesterone receptor (A and B) have been identified and separated by ion exchange chromatography (6–8). In the present studies receptors A and B were separated by DEAE-Sephadex chromatography (see *Materials and Methods*) and only the B form was used, since this is the more stable and more readily purified receptor component. Since receptors A and B are present in approximately equal amounts (6–8), some of the receptor loss during purification can be accounted for by the elimination of the A receptor. In very recent studies, to be reported elsewhere, we have purified the A form of the progesterone receptor by chromatography on ATP-Sepharose and DNA-cellulose. With preparations that are about 10% pure, we have observed PP_i-exchange activity that is comparable to that of the receptor B preparations used in the present study. This suggests that both forms of the progesterone receptor contain the enzymatic activity.

The two inhibitors, o-phenanthroline and rifamycin AF/013, have been of interest mainly because of their ability to block the activities of RNA and DNA polymerases (14–19). It is now known that these compounds also inhibit binding of the progesterone-receptor complex to nuclei in a cell-free system (2). In addition, o-phenanthroline has been shown to aggregate estrogen receptor from mouse mammary gland, indicating that this receptor may be a metalloprotein (26). While o-phenanthroline and rifamycin AF/013 are not highly specific inhibitors, the finding that they do block the PP_i-exchange activity offers additional evidence that the enzyme activity in question is due to progesterone receptor.

If the progesterone receptor contains an enzymatic activity as indicated here, this activity could represent a major event in the mechanism of steroid hormone action. The present study does not establish a complete enzymatic process, but simply illustrates a partial reaction involving the breakdown of ATP. However, this result should be considered together with the following properties of the receptor: (a) the receptor is believed to function at sites on the nuclear chromatin, closely associated to the synthesis of RNA; (b) the receptor is able to bind to polynucleotides (8, 27); (c) the binding of receptor to isolated nuclei and to ATP-Sepharose (28) is blocked by o-phenanthroline and it may, therefore, be a metalloprotein (2, 26); and (d) receptor binding to isolated nuclei and to ATP-Sepharose (28) is inhibited by rifamycin AF/013.

All of the above properties plus the PP_i-exchange activity are consistent with the possibility that the progesterone receptor is a nucleotidyltransferase or possibly a subunit or precursor to such an enzyme. The most notable enzymes of this class, DNA and RNA polymerases, are o-phenanthroline-sensitive metalloproteins, and many have been shown to be inhibited by rifamycin AF/013 (22, 29–31). Additional studies are needed to prove the identity of this catalytic function. However, the present results are very provocative and should stimulate additional interest in this approach toward understanding the mechanism of steroid hormone action.

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