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# INTERACTION OF CHICK OVIDUCT PROGESTERONE RECEPTOR WITH IMMOBILIZED AURINTRICARBOXYLIC ACID

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**Summary**—Aurintricarboxylic acid (ATA) was immobilized on Sepharose 4B via a carbodiimide coupling mechanism. A majority of the chick oviduct progesterone receptor was retained on the affinity resin and could be recovered upon washing the column with buffer containing free ligand or 3 M guanidine-HCl. The [$^3$H]progesterone–receptor complex retained its integrity following the chromatography on ATA-Sepharose as judged by sedimentation analysis. The procedure allowed significant purification of progesterone receptor: SDS-polyacrylamide gel electrophoresis of the purified preparation revealed elimination of many peptide bands present in the cytosol prior to ATA-Sepharose chromatography. The technique thus has a clear potential in characterization and purification of progesterone receptor.

## INTRODUCTION

Aurintricarboxylic acid (ATA), an effective chelating ligand, is known to inhibit functions of several nucleotidyl transferases [1, 2]. Previous work from this laboratory has demonstrated the inhibitory potential of ATA in several steroid receptor systems including rat liver glucocorticoid [3], avian oviduct progesterone [4, 5] and estradiol receptor from both rat uterus [6] and hen oviduct [7]. In the above studies, it was shown that addition of ATA blocks activation of steroid receptors as well as binding of activated receptors to acceptors such as target cell nuclei, DNA-cellulose and ATP-Sepharose. These observations [3–7] indirectly pointed to a direct action of ATA on the receptor molecule(s). In the present studies, we have employed affinity chromatography to examine unambiguously whether an interaction exists between ATA and the chick oviduct progesterone receptor, and if this technique can be used for receptor purification. A resin was prepared with ATA covalently linked to Sepharose 4B via amide linkage to aminohexane spacer groups.

## EXPERIMENTAL

### *Chemicals and materials*

All reagents were of analytical grade. Ultra-pure Tris and sucrose were from Schwarz/Mann (Orangeburg, NY); progesterone, monothioglycerol, ATP, sodium molybdate, Sepharose 4B (200–400 mesh), activated charcoal, phenylmethylsulphonyl fluoride (PMSF), ammonium salt of ATA and SDS-polyacrylamide gel electrophoresis high molecular weight calibration kit were purchased from Sigma Chemical Co. (St Louis, MO). [1,2-$^3$H]Progesterone (57.5 Ci/mmol) was obtained from New England Nuclear (Boston, MA). All buffers were prepared and stored at 4°C.

### *Synthesis of ATA-Sepharose*

ATA-Sepharose was synthesized via a carbodiimide coupling mechanism yielding an amide linkage between carboxylic equivalents of ATA and free amino groups of amino-hexane spacer arms covalently attached to Sepharose.

AH-Sepharose 4B (Pharmacia) (4 g) was swollen and washed with 0.5 M NaCl and with 0.1 M sodium acetate buffer (pH 4.5) and contained 6–10 $\mu$mol spacer groups/ml swollen resin. The resin was added to 50 ml of the above buffer containing 4.73 g (10 mmol) ATA trisodium salt (Aldrich). The pH was adjusted to 4.5 and 0.96 g (5 mmol) finely divided EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride] (Sigma) was added to the suspension over a 10 min period. The pH was adjusted to 4.5 with acetic acid and the temperature was maintained at 25–35°C. The mixture was allowed to shake for 24 h at 23°C and the resultant resin was washed first with buffer consisting of $NaHCO_3$ (0.2 M), 1 M NaCl, 10% dimethylsulfoxide, pH 8.3. The second wash buffer contained 0.1 M $CH_3COONa$, 1 N NaCl and 10% dimethyl sulfoxide, pH 4. Finally, the preparation was rinsed with deionized glass distilled $H_2O$. The resin was diluted 10-fold with Sepharose 4B prior to chromatography.

*Abbreviation:* ATA, 3-(*bis*(3-carboxy-4-hydroxyphenylmethylene)-6-oxo-1,4-cyclohexadene-1-carboxylic acid; SDS, sodium dodecyl sulfate; DES, diethylstilbestrol

Preparations used for chromatography contained about 1 mM ATA as determined by procedures described previously [5].

*Affinity chromatography*

Samples containing [$^3$H]progesterone–receptor complexes were chromatographed on 1–2 ml ATA-Sepharose columns. Tris buffer (50 mM Tris–HCl, 12 mM thioglycerol, 20% glycerol, pH 8.0) was used throughout the chromatographic procedures and contained ATA, KCl or $Na_2MoO_4$ where indicated. Guanidine HCl (3 M) was prepared in 10 mM Tris and pH was adjusted to 8.0. Columns were equilibrated with the above buffer containing 10 mM KCl and aliquots containing progesterone–receptor complexes were applied at a flow rate of 0.2 ml/min. The columns were washed with the same buffer and then with mobiles indicated in legends to figures. In each case 0.8–1.6 ml fractions were collected and the flow rate was maintained at 0.4 ml/min.

*Sedimentation rate analysis*

Linear 5–20% sucrose gradients (4.4 ml) were prepared in a buffer containing 10 mM Tris–HCl, 12 mM monothioglycerol, 1 mM EDTA, 20 mM $Na_2MoO_4$, 0.15 M KCl, pH 7.2 by using a Beckman gradient former. Samples were diluted with 10 mM Tris–HCl, pH 8 to lower glycerol concentration. The rate of sedimentation was determined using internal markers horseradish peroxidase (3.6S) and fungal glucose oxidase (7.9S) which were layered along with receptor samples. The gradients were centrifuged in a Sorvall TV-865 vertical rotor at 270,000 *g* for 2 h at 2°C. The gradients were fractionated by piercing the bottom of tubes.

*Gel electrophoretic analysis*

Aliquots from peak fractions obtained after ATA-Sepharose column chromatography were analyzed by SDS-polyacrylamide gel electrophoresis. Receptor samples were concentrated with 10% cold trichloroacetic acid with 20 $\mu$l of 1% (w/v) sodium deoxycholate per ml of sample at 0°C for 30–60 min. The pellets were dissolved in 50 $\mu$l of treatment buffer (125 mM Tris–HCl, 4% SDS, 20% glycerol, 1% $\beta$-mercaptoethanol, pH 6.8) and boiled for 3 min at 100°C in a boiling water bath. Electrophoresis was performed using a 10% linear (16 × 18 × 0.15 cm) polyacrylamide slab gel [11] for 11–12 h at 10 mA constant current per gel. The gels were silver stained according to Morrissey[12].

*Other methods*

Aqueous samples were combined with 5 ml scintillation fluid (4% spectra-flour [Amersham] in toluene), mixed thoroughly and cooled before counting in a Packard liquid scintillation counter with a 40% counting efficiency. Protein content in chromatographic samples was determined by the method of Bradford[13].

## RESULTS AND DISCUSSION

The affinity resin employed in the present studies was diluted with Sepharose to optimize the ligand concentration (0.6–1 mM) for adequate binding of the receptor with minimal dissociation of the complex. Figure 1 illustrates that the chick oviduct cytosol progesterone receptor can be retained on columns of ATA-Sepharose. Both non-activated and activated receptor forms showed comparable affinity for the resin allowing between 40–50% retention on ATA-Sepharose. Pre-treatment of cytosol preparations with free ligand abolished the binding of receptor to the affinity resin indicating that retention of receptor on the column involved ATA–receptor interaction. The binding of progestere–receptor complex to the affinity resin appeared to be of high affinity and could not be disrupted by 1 M KCl effectively. The receptor could be recovered in the elutes with 3 M guanidine HCl but this led to the dissociation of the complex and thus made it less suitable for further analysis.

The exact mechanism by which ATA interacts with the receptor is not known at present. Though polar and/or electrostatic interactions may exist between side chains of the protein and the keto, phenolic, or carboxylic groups of ATA, the above observations

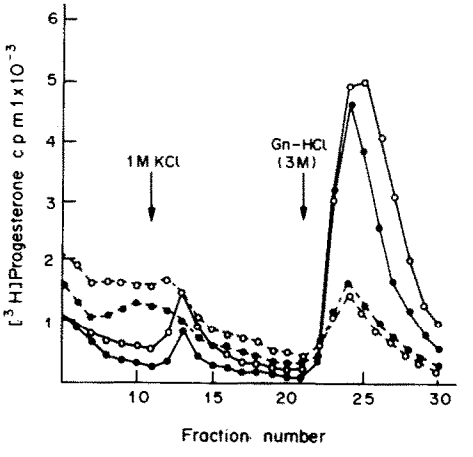


Fig. 1. Binding of [$^3$H]progesterone–receptor complex to ATA-Sepharose in the absence and the presence of free ligand. Chick oviduct cytosol was incubated with 20 nM [$^3$H]progesterone for 2 h at 0°C in a total volume of 0.5 ml. The steroid binding was monitored by the charcoal adsorption assay [9, 10]. Portions of the above cytosol preparation were kept at 23°C for 45 min for activation of the receptor. ATA (1 mM) or 10 mM Tris–HCl, pH 8, were added to the indicated samples 30 min prior to chromatography. Control and ATA-treated samples containing activated and non-activated [$^3$H]progesterone–receptor complexes were layered on separate affinity columns (2 ml). The columns were washed successively with 50 mM Tris–HCl, pH 8 containing 10 mM KCl, Tris-buffer containing 1 M KCl and finally with 3 M guanidine HCl. ●——●, non-activated progesterone–receptor complex (NA-PRc); ○——○, activated progesterone–receptor complex (A-PRc); ●——●, NA-PRc + ATA; ○——○, A + ATA. Fractions (1 ml) were collected and used for measurement of radioactivity.

(Fig. 1) indicated that the interaction may be primarily hydrophobic in nature. This interpretation is consistent with studies of ATA binding to serum albumin [14] indicating a strong protein–ligand interaction due to van der Waals forces. In addition, the ATA–receptor interaction may involve chelation of the ligand to a receptor-coordination metallic center. ATA is known to chelate a broad spectrum of metal cations [15, 16] and it has been suggested that steroid receptors may themselves be metalloproteins [17–19]. The observed ATA–receptor interaction may, therefore, arise by chelation with a prosthetic moiety of the receptor.

If ATA-Sepharose functions as an affinity resin, then the resin-bound progesterone–receptor complex should be readily eluted with a mobile phase containing the free ligand. Figure 2 illustrates the ability of 1 mM ATA to elute the resin-bound progesterone–receptor complex. This particular experiment yielded over 80% retention of the applied complex; 70% of which could be recovered by washing the column with 1 mM ATA. The relative ease and effectiveness of the use of ATA for receptor elution, thus, eliminated the employment of guanidine HCl for recovery of the receptor from the affinity columns.

Results from our previous studies had shown that the binding of steroid–receptor complexes to isolated nuclei, DNA-cellulose and ATP-Sepharose could be blocked by the addition of ATA to the receptor preparations either before or after receptor activation [4, 5, 20]. Although both non-activated and activated receptor forms were shown to be sensitive to ATA, the extent of inhibition was somewhat greater when ATA was added after activation of receptor complexes. Results of Fig. 1 further support the suggestion that both forms of progesterone–receptor complexes are ATA-sensitive as is shown by their retention on the ATA-Sepharose columns. The lengthy procedures of column chromatography, however, can activate the receptor due to dilution of cytosol and/or activation or elimination of certain other factor(s). This problem was addressed to in the present studies by use of sodium molybdate in our buffers in experiments that called for chromatography of the non-activated form of

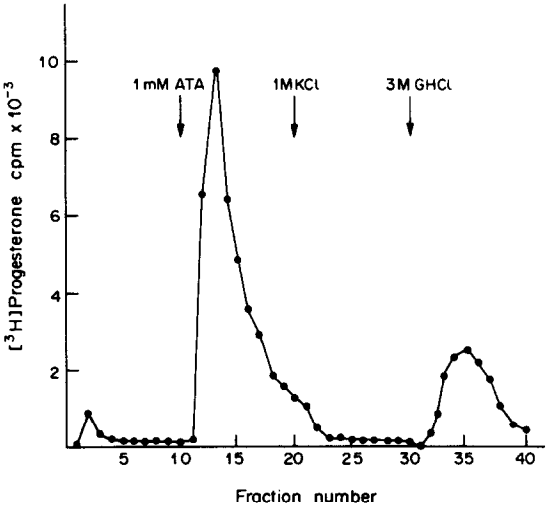


Fig. 2. Extraction of [$^3$H]progesterone–receptor complexes from ATA-Sepharose column by free ligand. Freshly prepared cytosol was incubated with 20 nM [$^3$H]progesterone for 2 h at 0°C and subsequently treated with equal volume (1:1, v/v) of Dextran-coated charcoal suspension to remove the free steroid. Portion (0.3 ml) of this cytosol was layered over 1 ml ATA-Sepharose column. Ten 1.2 ml fractions were collected each with Tris-buffer (10 mM Tris–HCl, 12 mM monothioglycerol, 20% glycerol, pH 8) containing 10 mM KCl, 1 mM ATA or 1 M KCl. Finally the column was washed with Tris-buffer containing 3 M GnHCl. Aliquots (500 $\mu$l) were used to measure the radioactivity.

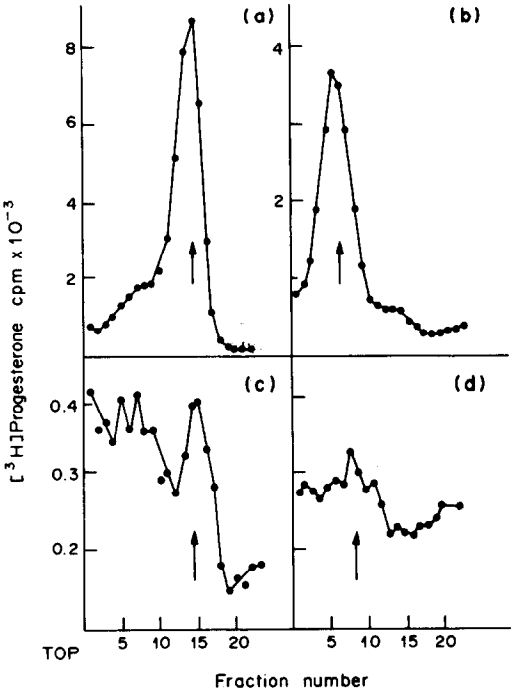


Fig. 3. Sedimentation rate analysis of activated and non-activated [$^3$H]progesterone–receptor complexes before and after ATA–Sepharose chromatography. Cytosol containing [$^3$H]progesterone–receptor complexes was divided into two groups; group I received 20 mM $Na_2MoO_4$ at 0°C while group II was incubated at 23°C for 1 h and then brought to 0°C. Portions of the cytosol from these groups were saved for the sedimentation analysis while the remaining cytosol was chromatographed on separate 1 ml ATA-Sepharose columns. For chromatography of the sample containing the non-activated receptor, the entire procedure was performed with buffers containing $Na_2MoO_4$. The receptor was eluted from the ATA-Sepharose columns with 1 mM ATA solution. Aliquots (200 $\mu$l) of above receptor preparations were used for determining the rate of sedimentation on 5–20% sucrose gradients. The position of arrows in panels a and c indicate the position of migration of the internal marker glucose oxidase (7.9S) while arrows in panels b,d show the position of the internal marker peroxidase (3.6S). a, cytosol +20 mM $Na_2MoO_4$; b, cytosol containing thermally activated receptor; c, molybdate-containing cytosol chromatographed on ATA-Sepharose; d, cytosol incubated at 23°C in the absence of molybdate and then passed over ATA-Sepharose column.

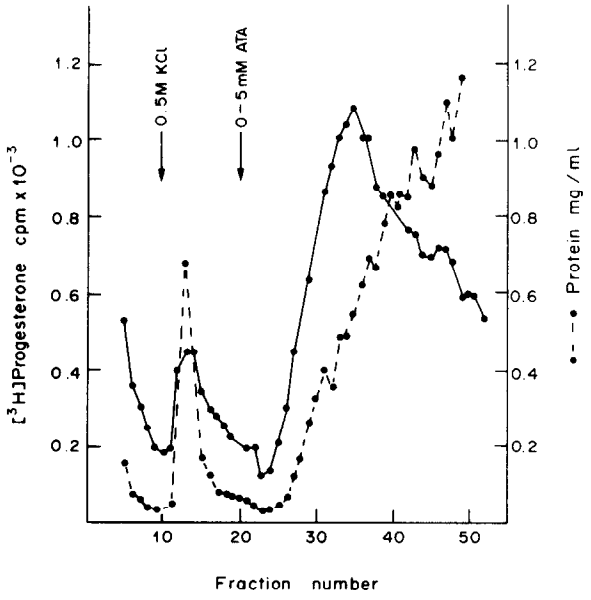


Fig. 4. Purification of [$^3$H]progesterone–receptor complex by ATA-Sepharose chromatography. Freshly prepared chick oviduct cytosol (1 ml) was incubated with 20 nM [$^3$H]progesterone for 2 h at 0°C and the resulting complexes were heat activated at 23°C for 1 h. Subsequently 0.8 ml aliquot of the above was layered over a 2 ml ATA-Sepharose column. The column was washed with Tris–HCl buffer containing 10 mM KCl, and 0.5 M KCl. Ten 1.2 ml fractions were collected. The remaining [$^3$H]progesterone–receptor complex was recovered by applying an ATA gradient (0–5 mM ATA). Fractions (1.2 ml) were collected on 0.5 ml aliquots were used for measuring radioactivity. Protein content in each fraction was determined according to Bradford[13]. ATA concentration in each fraction was determined spectrophotometrically as described [5].

progesterone–receptor complexes. This agent is being widely used to block the process of receptor activation and its stabilizes steroid receptors in the non-activated form when added before activation [21].

Parallel experiments, like one shown in Fig. 2 were performed by incubating the cytosol [$^3$H]progesterone–receptor complexes at 23°C for 1 h in the presence or absence of 20 mM $Na_2MoO_4$. We observed no difference in the extent of binding of receptor to the affinity resin in either case (not shown). To further confirm the identity of the chromatographed receptor, aliquots of peak fractions were analyzed for their rate of sedimentation in sucrose gradients. Results of Fig. 3 illustrate that the non-activated cytosol receptor sediments as an 8S moiety (Fig. 3a) which can be thermally transformed into a slower sedimenting 4S form (Fig. 3b) characteristic of activated progesterone–receptor complex [22]. Both the non-activated (Fig. 3c) and the activated (Fig. 3d) progesterone–receptor complexes remained in their respective molecular forms following the chromatography. The results of Fig. 3, therefore, suggest that both forms of progesterone receptor were retained by the ATA-Sepharose columns. Since activated receptor is apparently more sensitive to the inhibitory action of ATA, the processes which involve receptor–ATA interaction versus those which result in the inhibition of receptor uptake by the acceptors, may be mediated by different mechanisms. Alternatively, ATA in its free and the immobilized state may act via different mechanisms or sites on the receptor. Furthermore, the results of Fig. 3 demonstrate that the progesterone–receptor complex retains its integrity following ATP-Sepharose chromatography.

The potential of the use of ATA-Sepharose in the purification of receptor was also examined. Since the binding of receptor to the affinity resin appears to be strong, the columns can be washed with high-salt buffer solution without much loss of receptor (Fig. 4). The resin-bound receptor can be obtained by employing a gradient of ATA solutions. The peak fraction in this experiment was eluted with about 1 mM ATA. We have observed that the receptor purified by ATA-Sepharose chromatography becomes quite unstable which leads to loss of steroid from the complex. This contributes to underestimation of the purification that can be achieved by this method. Although only 20–50-fold purification is obtained by this procedure when calculated based on the radioactivity/mg protein; the receptor appears to be purified to a greater extent. The SDS-electrophoresis of the cytosolic and purified receptor preparations revealed elimination of several peptide bands following the affinity chromatography (not shown). Further studies are in progress to establish with precision the potential of ATA-Sepharose in the purification of progesterone receptor.

In conclusion, the results of this study show that ATA interacts directly with the progesterone receptor and that use of ATA-Sepharose chromatography leads to significant purification of the receptor. Al-

though extraction of receptor from ATP-Sepharose by use of free ATA has been demonstrated [4], the reverse was not possible (not shown). In our preliminary studies (not shown) incubation of receptor with calf thymus DNA reduced somewhat that binding of receptor to ATA-Sepharose suggesting that DNA and ATA binding sites of receptor are either related or are in close proximity. Ineffectiveness of free ATP in eluting receptor from ATA-Sepharose clearly suggests that the interaction between ATA and receptor is of higher affinity than that which exists between ATP and the receptor.

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