Receptor Interconversion Model of Hormone Action. 1. Purification of a Factor Involved in Conferring Estradiol Binding Properties to the Estrogen Receptor†

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ABSTRACT: We previously demonstrated that the chick oviduct estrogen receptor exists in three interconvertible forms. Two of these forms bind estradiol with high but distinct affinities. A third form exists as a non-estrogen binding recyclable form, R_{nb}, which upon treatment with ATP/Mg^{2+} is quantitatively converted to the lower affinity estradiol binding form. We now describe the isolation from chick oviduct cytosol of a factor involved in this conversion and its 1100-fold purification by ammonium sulfate fractionation, DEAE ion-exchange chromatography, and size-exclusion HPLC. The factor elutes from the size-exclusion column with an apparent molecular weight of 40 000. This highly purified factor potentiates estradiol binding in a dose-dependent manner in the presence of ATP/Mg^{2+}. Its activity is destroyed by heating or by trypsin treatment but is relatively stable to freezing and thawing and is inert to treatment with reducing agents. ATP is an essential nucleotide substrate; GTP and cyclic nucleotides are inactive. Studies of cation dependence demonstrate that Mg^{2+} is also essential; Ca^{2+} alone is completely ineffective in catalyzing receptor potentiation and does not synergize with Mg^{2+}. In the presence of excess ATP/Mg^{2+} and a fixed concentration of F_y, the K_m for potentiation of estradiol binding is approximately 0.4 nM.

The chick oviduct system is proving to be an excellent model for the study of estrogen action. Depending upon the degree of estrogenic stimulation, it is possible to identify two high-affinity estrogen binding receptors that are characterized by a number of clearly defined properties. These two forms, R_x and R_y, possess equilibrium dissociation constants (K_d's) of 0.06 and 0.8 nM and sedimentation constants of 4.2 and 3.5 S, respectively (McNaught & Smith, 1986; McNaught et al., 1986). Evidence from our laboratory suggests that they have different functional roles in promoting estrogen action such that the purified R_x specifically enhances RNA polymerase II activity (Taylor & Smith, 1982), while the lower affinity R_y species appears to have a prerequisite role in estrogen-dependent gene transcription (Smith & Taylor, 1981; Taylor & Smith, 1985). The R_x conformation is also preferentially stabilized by the anti-estrogen hydroxytamoxifen (McNaught et al., 1986).

Estrogen stimulation of the chick oviduct followed by short periods of withdrawal from estrogen results in the appearance of a non-estradiol binding form (R_{nb}) of the receptor in cytosol. The presence of this form can be detected indirectly by treatment of cytosol with ATP/Mg^{2+}. This treatment quantitatively converts the receptor into an estrogen binding form (R_y) allowing it to be assayed by [^{3}H]estradiol binding (Raymoure et al., 1985). Further studies investigating receptor potentiation in crude cytosol delineated the nucleotide and cation requirements of the system (Raymoure et al., 1986). The authenticity of R_y obtained by potentiation was indicated by virtue of its K_d, sedimentation coefficient, steroid specificity, immunoactivity, and its molecular weight of 66 000 (Raymoure et al., 1986). Since these data were obtained from studies with unfractionated cytosol, it became incumbent upon us to demonstrate R_{nb} to R_y conversion in a more defined system and to determine more clearly the nature of the ATP/Mg^{2+}-dependent regulatory factor(s).

In this report, we describe the purification of an estrogen receptor potentiating factor, which we defined as F_y because of its role in converting R_{nb} to R_y. By use of ammonium sulfate fractionation, DEAE Bio-Gel A chromatography, and HPLC gel permeation chromatography, a 1100-fold purification was achieved. The demonstration that highly purified F_y has serine kinase activity, and the relationship between phosphorylation/dephosphorylation reactions and acquisition of estrogen binding by the receptor, is the subject of the second of three papers in this issue (Dayani et al., 1990).

Experimental Procedures

Chemicals. All agents used were of analytical grade or equivalent. Ammonium sulfate, EDTA, hydrochloric acid, Norit-A charcoal, and sodium chloride were purchased from Fisher Scientific (Pittsburgh, PA). Dextran T70 was supplied by Pharmacia (Piscataway, NJ), and Tris base came from Boehringer Mannheim (Indianapolis, IN). ADP, ATP, GDP, GTP, diethylstilbestrol (DES),[1] monothioglycerol, sodium molybdate, trypsin, and trypsin inhibitor were purchased from Sigma (St. Louis, MO), and Bio-Gel A-0.5M and DEAE Bio-Gel A were from Bio-Rad (Richmond, CA). 17β-Estradiol was obtained from Steraloids (Wilton, NH), while [2,4,5,7-^{3}H]estradiol (100 Ci/mmol) was purchased from Amersham (Arlington Height, IL).

Hormone Treatment. White Leghorn chicks (Texas Animal Specialties, Humble, TX), aged 7–8 days, were treated with daily subcutaneous injections of 2 mg of DES dissolved in sesame seed oil for a 2-week period. The animals were then

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[1] Abbreviations: DES, diethylstilbestrol; SDS, sodium dodecyl sulfate.

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withdrawn from the estrogenic stimulation prior to sacrifice.

Preparation of Cytosol. Chick oviduct tissue, immediately after collection, was rinsed in ice-cold saline, weighed, minced, and then homogenized with 5 volumes (w/v) of TESH buffer (10 mM Tris-HCl, 1.5 mM EDTA, and 12 mM monothioglycerol, pH 7.4 at 24 °C) using a Polytron tissue homogenizer (Brinkmann, Westbury, NY) with 3 × 5 s pulses. The homogenate was first centrifuged at 5000*g* for 20 min; the resultant supernatant was subjected to additional centrifugation at 105000*g* for 90 min. Fatty material that collected at the surface was carefully aspirated off, leaving a clear supernatant.

Ammonium Sulfate Fraction. The non-steroid binding form of the chick oviduct estrogen receptor was isolated from cytosol by adding a saturated solution of ammonium sulfate (prepared in TESH buffer, pH 7.4) until a final concentration of 30% ammonium sulfate was attained. The mixture was stirred for an additional 30 min and the suspension sedimented by centrifugation for 20 min at 10000*g*. The resulting pellets were carefully rinsed with TESH buffer, after which they were redissolved in an appropriate volume of TESH buffer. This partially purified estrogen receptor solution containing predominantly R_{nb} was spun at 105000*g* for 30 min to sediment any remaining insoluble material. The factor which converted the nonbinding form of the receptor to its lower affinity binding form was similarly isolated in subsequent ammonium sulfate fractions as summarized under Results.

Steroid Binding Assays. Saturation analysis of the cytosolic-derived estrogen receptor was performed in the presence of [^{3}H]estradiol at final concentrations ranging from 80 pM to 10 nM. Single-point assays, where applicable, used 1.2 nM [^{3}H]estradiol. Nonspecific binding was quantitated by the addition of a 100-fold molar excess of radioinert DES to duplicate assay tubes. All experiments were carried out in the presence of 10 mM Na_2MoO_4, and additional reagents were prepared as concentrated stock solutions in TESH buffer adjusted to pH 7.4. Incubations were generally performed under exchange conditions of 3 h at 30 °C (Taylor et al., 1980). In some specific cases, the 30 °C incubation was followed by overnight incubation at 4 °C for 18 h. Dextran-coated charcoal adsorption assays were used to measure total and nonspecific bound [^{3}H]estradiol, with saturation assays being terpreted by Scatchard analysis (Scatchard, 1949).

Ion-Exchange Chromatography. Minicolumns were prepared containing a 2–5-mL bed volume of Bio-Rad DEAE Bio-Gel A. The resin was extensively washed with elution buffer prior to use. Soluble protein solutions containing F_y were then eluted at 4 °C from the column either with linear gradients of 0–400 mM NaCl in TESH buffer or by stepwise NaCl elutions with varying salt concentrations (typically 0, 50, 100, 200, and 300 mM).

HPLC Chromatography. Fractions containing activating factor F_y were injected into a TSK-G3000-SW column (LKB, Bromma, Sweden) equilibrated at 4 °C with a TESH buffer containing 250 mM NaCl. Samples were eluted under isocratic conditions with a flow rate maintained at 0.5 mL/min. Individual fractions were subsequently assayed for F_y activity. The F_y activity peak was then reapplied to the HPLC gel permeation column for additional purification.

Polyacrylamide Gel Electrophoresis. Purified F_y was prepared for electrophoresis by boiling in gel loading buffer for 5 min at 100 °C. The samples were then loaded on 10% Porzio and Pearson (1977) slab gels and developed under constant current conditions of 25 mA for approximately 8 h. Proteins were visualized by Coomassie Blue or silver staining depending on the concentration of protein initially loaded.

Protein Assay. Protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

Results

Isolation of the Factor (F_y) Which Converts the Non-Steroid Binding Form of the Receptor (R_{nb}) to an Estrogen Binding Form (R_y). Oviduct cytosol from estrogen-stimulated chicks withdrawn from estrogen treatment for 1–2 days contained a minor amount of the lower affinity estrogen binding receptor R_y, together with an excess of the non-steroid binding form R_{nb} (Raymoure et al., 1985, 1986). Since ammonium sulfate fractionated receptor (30%) was devoid of the factor (F_y) which converted R_{nb} to R_y, the ammonium sulfate supernatant was tested for its ability to confer steroid binding activity to the receptor. Reconstitution of the 30% ammonium sulfate pellet with an aliquot of the supernatant and treatment with ATP/Mg^{2+} gave identical augmentation of estradiol binding to that observed following treatment of the original cytosol with ATP/Mg^{2+} (data not shown).

Fractionation of the ammonium sulfate supernatant in 10% increments beyond 30% revealed that the majority (>85% of total F_y activity) was contained within the 40–50% and 50–60% fractions. Therefore, a 40–60% ammonium sulfate fraction was used routinely as the starting material for subsequent purification steps. Indeed, using this fraction, it was possible, in a concentration-dependent manner, to produce the same maximal level of R_y augmentation as observed in cytosol.

Molybdate was included in the receptor potentiation reactions to help stabilize R_y; however, it was subsequently found that the conversion of R_{nb} to R_y occurred with similar efficiency in the absence of molybdate.

The role of estradiol in the conversion of R_{nb} to R_y was investigated. Figure 1A shows a typical Scatchard analysis of F_y-mediated conversion of R_{nb} to R_y as illustrated by the appearance of high-affinity, saturable [^{3}H]estradiol binding. This conversion occurred within 3 h at 30 °C but not at 4 °C. To investigate whether this conversion would occur in the absence of estradiol at 30 °C R_{nb} was incubated with F_y in the presence of ATP/Mg for 3 h at 30 °C; then the receptor was labeled with increasing concentrations of [^{3}H]estradiol for 18 h at 4 °C (Figure 1B). It is clear from a comparison of panels A and B of Figure 1 that the same amount of augmentation of estradiol binding is obtained when conversion is induced in the absence of estradiol at 30 °C.

The substrate used to monitor subsequent purification of F_y was R_{nb} obtained by precipitation at 30% ammonium sulfate. The 40–60% ammonium sulfate containing F_y was dialyzed overnight against TESH buffer to remove residual ammonium sulfate. This preliminary step resulted in precipitation of protein. After centrifugation, the supernatant was assayed and found to have maintained F_y activity. Dialysis therefore effected a partial purification (Table I).

Aliquots of postdialysis supernatant were chromatographed with DEAE Bio-Gel A using a 0–0.4 M NaCl salt gradient. Each fraction was incubated with ammonium sulfate purified R_{nb} in the presence of ATP/Mg^{2+} at 30 °C. Fractions containing F_y were detected by their capacity to confer estradiol-specific binding to R_{nb}. F_y was isolated in fractions eluting between 0.15 and 0.2 M NaCl. Ion-exchange chromatography was repeated using a TSK-DEAE-5PW HPLC column eluted with a 0–400 mM linear NaCl gradient. Again, each chromatographic fraction was assayed for its ability to convert R_{nb} to R_y. F_y was eluted at 180 mM NaCl. However, recoveries of F_y activity for this HPLC ion-exchange method were very

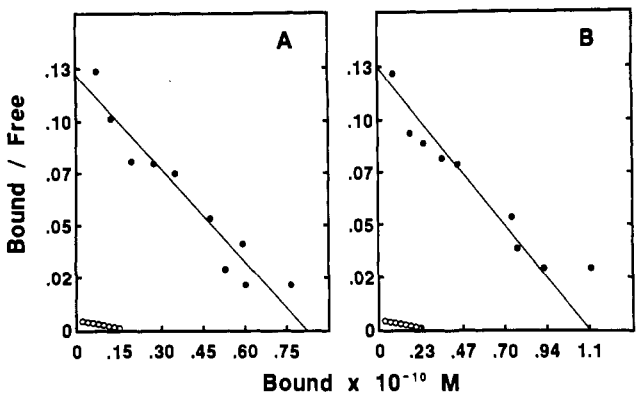

FIGURE 1: Conversion of the nonbinding form of chick estrogen receptor to R_y in the presence of F_y and the effect of estrogen. Oviduct cytosol was prepared from DES-stimulated chicks that were withdrawn from DES treatment for 2 days. Cytosol was fractionated using saturated ammonium sulfate into a 30% fraction containing receptor and a 40–60% fraction containing F_y activity. The respective precipitated fractions were redissolved in a volume equivalent to one-fourth volume of the cytosol from which the fractions were prepared. For Scatchard analysis, a 200-μL receptor fraction and a 20-μL F_y fraction were used in duplicate for each data point. Binding assays using a 80 pM–10 nM range of [^{3}H]estradiol concentration were performed by incubating at 30 °C for 3 h in the presence of F_y and 5 mM ATP/Mg^{2+}, and then incubating for an additional 18 h at 4 °C before being charcoal-treated to separate unbound steroid. Nonspecific binding was accounted for with duplicate assays in the presence of a 100-fold molar excess of unlabeled DES. Concurrently, a similar assay was run, but during the incubation at 30 °C for 3 h, estrogen was omitted. Then, varying concentrations (80 pM–10 nM) of [^{3}H]estradiol were added, and the assay was continued for 18 h at 4 °C. Resultant data were interpreted by the method of Scatchard. (Panel A) Test assay with 5 mM ATP/Mg and 10 mM molybdate in the absence (○) or presence (●) of F_y. (Panel B) As per panel A, but in the absence of estradiol for the 30 °C incubation.

Table I: Purification of Chicken Oviduct Estrogen Receptor Potentiating Factor (F_y)[a]

purification step	F_y activity (μg of protein)	purification (x-fold)	yield (%)
none	920	0	
$(NH_4)_2SO_4$	170	5.4	100
dialysis	104	8.8	100
DEAE Bio-Gel A	45	20.4	88
TSK 3000	2	460	20
TSK 3000	0.8	1150	8

[a] Standardized binding assays to determine the level of F_y activity were performed as described under Experimental Procedures. In brief, 200 μL of $(NH_4)_2SO_4$-precipitated R_{nb} receptor substrate was incubated with 1.2 nM [^{3}H]estradiol, 2 mM ATP, and 5 mM Mg^{2+} for 3 h at 30 °C. F_y activity at each purification step is expressed as the amount of protein required to give comparable conversion of R_{nb} to R_y using estradiol-specific binding as an end point.

low; therefore, an alternative purification protocol was sought.

The F_y-containing eluent fraction from DEAE Bio-Gel A was directly injected onto a TSK-G3000 HPLC column which had been calibrated with molecular weight standards. Each fraction was again assayed with R_{nb} + ATP/Mg^{2+}. Under isocratic conditions, F_y was eluted as a single peak of activity with a recovery of 20% (Figure 2A, Table I). Scatchard analysis showed that the TSK3000-purified F_y had similar activity to ammonium sulfate purified F_y (Figure 3). These data confirmed that HPLC-purified F_y was able to quantitatively convert R_{nb} to R_y. Previous studies in cytosol showed that conversion of R_{nb} to R_y was quantitative (Raymoure et al., 1985). This is an important property since quantitation of R_{nb} is necessary for kinetic studies.

Partially purified F_y was reapplied to the TSK-G3000 column for additional purification. The rechromatographed

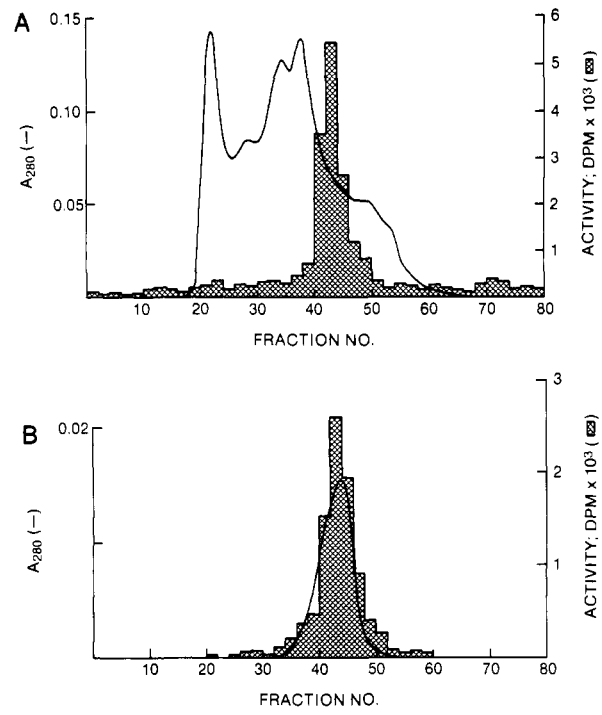

FIGURE 2: Preparative gel permeation HPLC chromatography. Partially purified F_y samples from DEAE ion-exchange chromatography were chromatographed on a 8 × 300 mm TSK-G3000-SW column eluted under isocratic conditions with a TESH-based buffer containing 250 mM NaCl. Individual fractions were analyzed for F_y activity by using the standardized receptor assay comprising ammonium sulfate purified R_{nb}, 1.2 nM [^{3}H]estradiol, 2 mM ATP, and 5 mM Mg^{2+} incubated at 30 °C for 3 h. Nonspecific binding was assessed with parallel assays containing a 100-fold molar excess of radioinert DES. (Cross-hatched area) Specific estradiol binding. (—) Absorbance of eluate at 280 nM. (Panel A) First passage of F_y through TSK-G3000–SW column. (Panel B) Second passage of F_y through same column.

F_y fraction appeared as a single sharp protein peak eluting at a molecular weight of approximately 40K (Figure 2B). The level of purification was approximately 1100-fold (Table I). SDS-polyacrylamide gel electrophoresis revealed two bands of molecular weight 40K and 45K (Figure 4). The 45 000-dalton band was identified as being ovalbumin in a series of one- and two-dimensional gels using authentic standard proteins as a reference. The 40-kDa band stained extremely weakly with silver and could not be reliably observed in 2D gels. Attempts to remove residual concentrations of endogenous ovalbumin from the purified HPLC samples met with limited success due to poor recovery of F_y when subjected to further purification.

It should be stressed that HPLC purification using either gel exclusion or ion-exchange chromatography would only provide active F_y when glass rather than stainless-steel columns were used.

Properties of Purified Chick Oviduct F_y. The stability of F_y was evaluated. Its activity was destroyed by boiling the purified protein for 5 min (Table II). Furthermore, treatment with trypsin followed by addition of trypsin inhibitor, prior to incubation with R_{nb} and ATP/Mg^{2+}, demonstrated that at a trypsin concentration of 20 μg/mL 35% of F_y activity was destroyed. At a trypsin concentration of 100 μg/mL, the activity of F_y was completely destroyed. Control assays demonstrated that F_y activity was preserved if trypsin activity was blocked by trypsin inhibitor (Table II). The effect of a sulfhydryl reducing agent, dithiothreitol, was tested at concentrations of 0.1–1 mM. This reagent alone or with F_y was unable to convert R_{nb} to R_y. Furthermore, it had no inhibitory

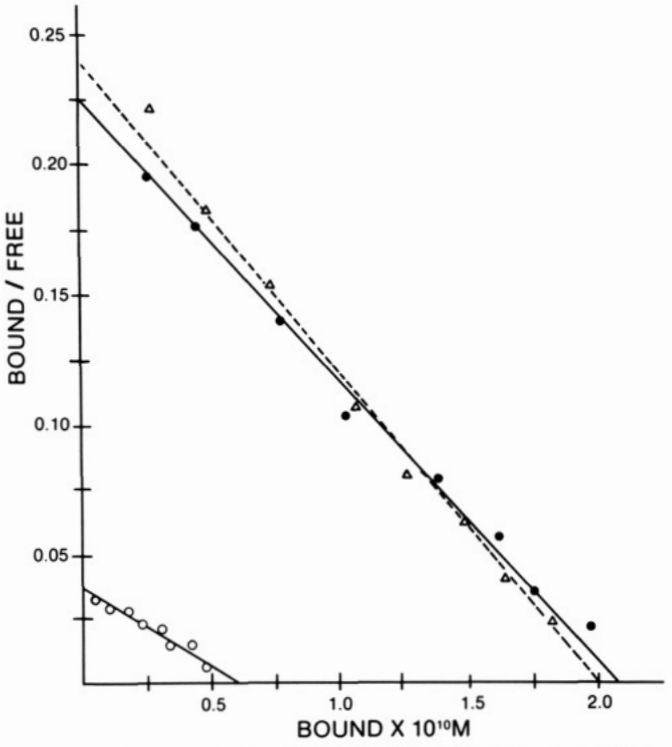

FIGURE 3: Activation of R_{nb} after reconstitution with F_y. Ammonium sulfate fractionated chick oviduct estrogen receptor containing predominantly R_{nb} was incubated in the presence of 2 mM ATP and 5 mM $MgCl_2$ for 3 h at 30 °C with varying concentrations (80 pM–10 nM) of [^{3}H]estradiol. Nonspecific binding was quantitated with the addition of a 100-fold molar excess of radioinert DES, and the resultant data were analyzed by Scatchard analysis: (O—O) control assay incubated in the absence of F_y; (●---●) test assay incubated in the presence of ammonium sulfate isolated F_y (25 μL; 170 μg of protein); (Δ---Δ) test assay incubated with HPLC-purified F_y (50 μL; 2 μg of protein).

Table II: Stability of Chick Oviduct Estrogen Receptor Activating Factor[a]

treatment	F_y activity (%)
control	100
heat (100 °C, 5 min)	9
trypsin (20 μg/mL; 30 °C, 60 min)	65
trypsin + trypsin inhibitor (20 μg/mL; 30 °C, 60 min)	97
trypsin (100 μg/mL; 30 °C, 60 min)	2
trypsin + trypsin inhibitor (100 μg/mL; 30 °C, 60 min)	92

[a] Standardized binding assays were performed as described under Experimental Procedures. Fifty microliters of HPLC-purified F_y was preincubated for 60 min at 30 °C in the absence or presence of the various reagent(s). In the trypsin experiments, to block the effect of trypsin on R_{nb}, trypsin inhibitor was added just prior to assaying for the activity in converting R_{nb} to R_y.

or stimulatory effect on F_y activity (data not shown).

Nucleotide concentration dependence using HPLC-purified F_y was examined in detail. Parallel experiments were undertaken with ADP, ATP, GDP, and GTP. As illustrated in Figure 5, there is a requirement for ATP, with essentially optimum activity being achieved between 0.5 and 2.0 mM nucleotide. ADP was able to effect a degree of augmentation of R_y, albeit substantially attenuated compared to ATP, while GDP and GTP were ineffectual.

The dose dependence of the conversion of R_{nb} to R_y in the presence of excess ATP/Mg^{2+}, illustrated by Figure 6, demonstrates that maximal potentiation of estradiol binding is accomplished with approximately 1 μg of protein. We previously showed that conversion of R_{nb} to R_y is quantitative

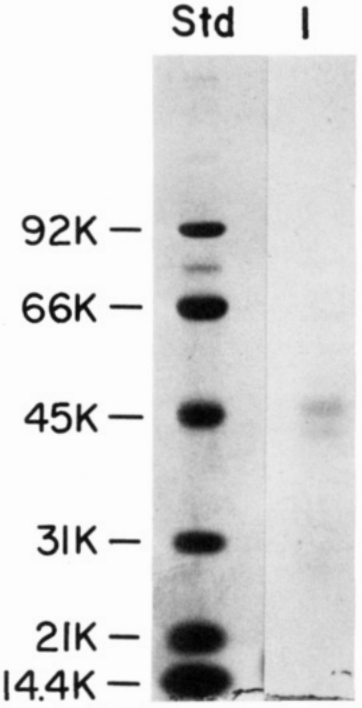

FIGURE 4: Polyacrylamide gel electrophoresis of HPLC-purified F_y. Samples of the purified potentiating factor were prepared for electrophoresis by boiling in gel loading buffer containing 5% mercaptoethanol. They were then resolved in 10% Porzio and Pearson slab gels for approximately 8 h under constant current conditions of 25 mA (Porzio & Pearson, 1977). Proteins were detected by Coomassie blue staining. Protein standards were run concurrently and comprised lysozyme (14 000), soybean trypsin inhibitor (21 000), carbonic anhydrase (31 000), ovalbumin (45 000), bovine serum albumin (67 000), phosphorylase B (93 000), β-galactosidase (116 000), and myosin (200 000). Lane 1, protein standard. Lane 2, HPLC-purified F_y.

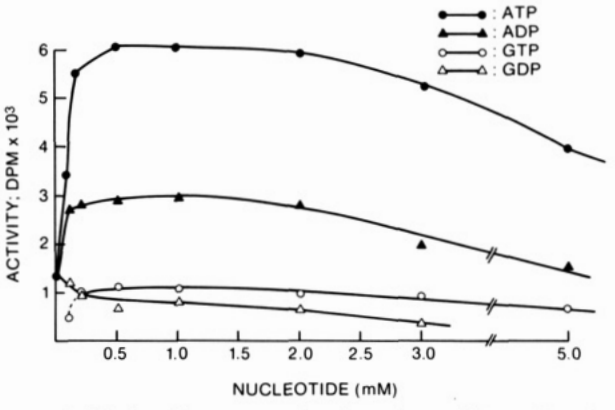

FIGURE 5: Nucleotide concentration dependence of F_y-mediated R_y augmentation. The analyses were performed using HPLC-purified F_y and ammonium sulfate isolated R_{nb}. Single-point assays using 1.2 nM [^{3}H]estradiol were carried out at 30 °C for 3 h in triplicate using 5 mM Mg^{2+} and varying concentrations of ATP (●), ADP (▲), GTP (O), or GDP (Δ). Nonspecific binding was quantitated with a 100-fold molar excess of radioinert DES.

under these experimental conditions (Raymoure et al., 1985). It was therefore possible to indirectly quantitate the concentration of R_{nb} in the receptor preparation. Varying concentrations of R_{nb} were incubated with ATP/Mg^{2+}. The results were analyzed by using a Lineweaver–Burk double-reciprocal plot. The Michaelis consstant (K_m) for the purified chick oviduct F_y-mediated R_{nb} to R_y conversion as extrapolated from the linear plot was approximately 0.4 nM (data not shown).

A specific requirement for Mg^{2+} ions was previously described for R_y augmentation in cytosol (Raymoure et al., 1986). Auricchio and his colleagues using a mammalian

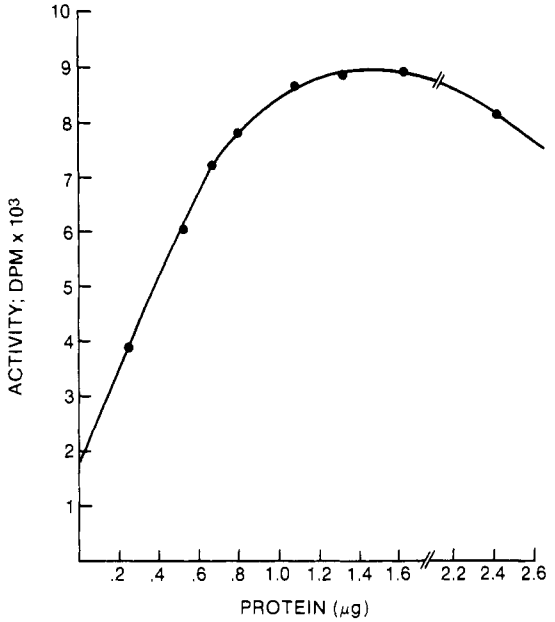

FIGURE 6: Dose response of F_y-mediated receptor augmentation. Duplicate assays were performed using 1.2 nM [^{3}H]estradiol to label the potentiated receptor. Varying concentrations of the purified activating factor were incubated with 2 mM ATP, 5 mM Mg^{2+}, and ammonium sulfate purified R_{nb} for 3 h at 30 °C. Nonspecific binding was determined in the presence of a 100-fold molar excess of radioinert DES.

estrogen receptor potentiating system were able to demonstrate that both Mg^{2+} and Ca^{2+} were effective individually and were synergistic in converting the receptor to its estrogen binding form (Auricchio et al., 1981). Accordingly, comparative experiments were performed using pure F_y. The F_y activity purified according to our protocl was compared to similar activity purified according to Auricchio et al. (1981). Chick oviduct cytosol was used as the source of potentiating activity for both purification protocols. Cation concentration dependence was evaluated. Irrespective of concentrations of Ca^{2+} used, or the method of isolation, F_y purified from chick oviduct cytosol was unable to conver R_{nb} to R_y in the absence of Mg^{2+} (Figure 7); moreover, synergism between Mg^{2+} and Ca^{2+} could not be demonstrated.

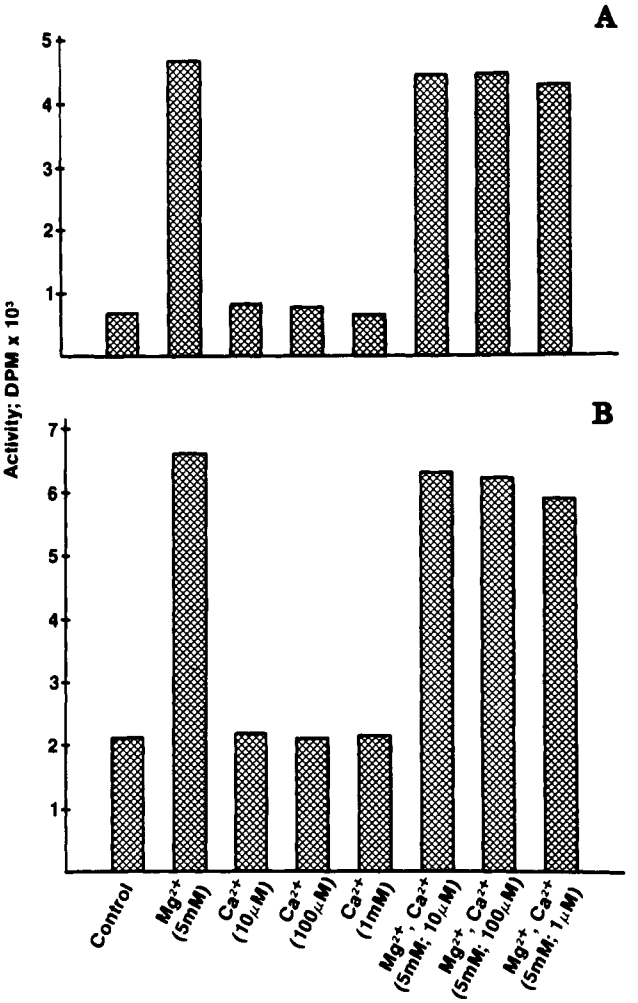

FIGURE 7: Cation concentration dependence of receptor augmentation by HPLC-purified F_y. F_y was prepared as described under Experimental Procedures and by the method of Auricchio et al. (1981). Standardized binding assay experiments were carried out in duplicate using various concentrations of Mg^{2+} and Ca^{2+} individually or together, along with 1.2 nM [^{3}H]estradiol, 2 mM ATP, receptor (R_{nb}), and the two comparative F_y sources. a 100-fold molar excess of radioinert DES added to parallel tubes was used to quantitate nonspecific binding. (Panel A) Experiments using HPLC-purified F_y. (Panel B) Using partially purified F_y according to the protocol of Migliaccio et al. Activity is expressed as specific [^{3}H]estradiol binding.

Discussion

The observation that steroid receptors in target tissues are present but are not detected because they lack the ability to bind ^{3}H-steroid ligands is important to our understanding of steroid hormone action. In 1968, Munck and Brinck-Johnson demonstrated that ATP concentrations and glucocorticoid binding decreased in parallel when thymocytes were incubated under nitrogen. When these cells were then incubated under aerobic conditions, ATP concentrations increased and glucocorticoid binding was regained (Munck & Brinck-Johnson, 1968). It was later shown that the receptors which had lost the capacity to bind glucocorticoids were bound to nuclei of WEH1-7 cells and that these were present in amounts comparable to that of receptors in normal cells (Mendel et al., 1986). These more recent data suggest a functional role for the non-steroid binding receptor form and suggest that cells which have been considered receptor deficient may indeed contain a non-steroid binding form of the receptor.

It has been proposed, because of the association of steroid binding with increased ATP concentrations, that acquisition of steroid binding is a result of receptor phosphorylation. Indeed, Auricchio's work argues that tyrosine phosphorylation of the mammalian estrogen receptor is required to induce estradiol binding capability (Auricchio et al., 1981; Migliaccio et al., 1984, 1986). However, other investigators have shown that pure receptors can be phosphorylated using [γ-^{32}P]ATP in the absence of exogenous kinases (Miller-Diener et al., 1985; Baldi et al., 1986). Since these studies utilized steroid binding forms of the receptors, it became difficult to conclusively prove that acquisition of steroid binding is a direct consequence of receptor phosphorylation rather than some other ATP-mediated effect resulting in a change in receptor conformation. For example, data have been presented which argue that the steroid binding activity might not be associated with phosphorylation and that a change in redox state is involved (Housley et al., 1982). To begin to resolve these issues, it is necessary to isolate and purify the factors involved in the conversion of a receptor from its non-steroid binding to its binding form.

We have previously shown that treatment of chick oviduct cytosol with ATP/Mg^{2+} caused potentiation of the lower affinity estrogen receptor (R_y) and have defined this process as the conversion R_{nb} to R_y, where R_{nb} is the estrogen receptor in its non-estradiol binding form (Raymoure et al., 1985, 1986). We have now described separation of this activity from

the estrogen receptor by ammonium sulfate fractionation. Substantial purification of this factor (F_y) has been achieved by DEAE ion-exchange chromatography and size-exclusion HPLC. The activity of F_y is dependent upon ATP/Mg^{2+} as substrate. Potentiation occurs equally well in the presence or absence of estradiol and molybdate (10 mM). In contrast to a similar factor partially purified by Auricchio's group (Auricchio et al., 1981) from mammalian sources, Ca^{2+} is completely inactive in the chick oviduct system. Highly purified F_y in a dose-dependent manner converts R_{nb} to R_y. By varying the concentration of R_{nb} and using a concentration of ATP/Mg^{2+} that is not rate limiting, we have shown that the K_m of the reaction is approximately 0.4 nM.

F_y is proteinacious in nature since its activity is destroyed by heat and trypsin treatment; it is resistant to DNase and RNase. Its activity is neither stimulated nor inhibited by dithiothreitol. Furthermore, dithiothreitol cannot substitute for F_y or for ATP/Mg^{2+}; it is therefore distinct from thioredoxin (Grippo et al., 1983). On the basis of these observations, the F_y-mediated conversion of R_{nb} to R_y is unlikely to be caused simply by reduction of disulfide bonds. The fact that the action of F_y requires Mg^{2+} and a hydrolyzable γ-phosphoryl moiety of ATP (Raymoure et al., 1985, 1986) suggests that an ATP-dependent kinase and/or phosphatase is involved in the R_{nb} to R_y conversion.

In Dayani et al. (1990), we demonstrate that the serine kinase activity manifested by HPLC-purified F_y is not sufficient to convert R_{nb} to R_y in the absence of other factors. It appears that both serine kinase and serine phosphatase activities are required. Both of these activities appear to be essential for the conversion of the chick oviduct estrogen receptor from its non-estrogen binding into its steroid binding form (Dayani et al., 1990). Since steroid binding plays a crucial role in the activity of receptors as transcription factors, the enzymes regulating the steroid binding state may play a primary role in the control of transcription of estrogen-responsive genes.

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Registry No. ATP, 56-65-5; Mg, 7439-95-4; F_y kinase, 93792-06-4; estradiol, 50-28-2.

References

Auricchio, F., Migliaccio, A., Lastoria, G., Lastoria, S., & Schiavone, E. (1981) *Biochem. Biophys. Res. Commun. 101*, 1171–1178.

Baldi, A., Boyle, D. M., & Wittliff, J. L. (1986) *Biochem. Biophys. Res. Commun. 135*, 597–606.

Bradford, M. (1976) *Anal. Biochem. 72*, 248.

Dayani, N., McNaught, R. W., Shenolikar, S., & Smith, R. G. (1990) *Biochemistry* (second of three papers in this issue).

Grippo, J. F., Tienrungroj, W., Dahmer, M. K., Housley, P. R., & Pratt, W. B. (1983) *J. Biol. Chem. 258*, 13658–13668.

Housley, P. R., Dahmer, M. K., & Pratt, W. B. (1982) *J. Biol. Chem. 257*, 8615–8618.

McNaught, R. W., & Smith, R. G. (1986) *Biochemistry 25*, 2073–2081.

McNaught, R. W., Raymoure, W. J., & Smith, R. G. (1986) *J. Biol. Chem. 261*, 17011–17017.

Mendel, D. B., Bodwell, J. E., & Munck, A. (1986) *Nature 324*, 478–480.

Migliaccio, A., Rotondi, A., & Auricchio, F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5921–5925.

Migliaccio, A., Rotondi, A., & Auricchio, G. (1986) *EMBO J. 5*, 2867–2872.

Miller-Diener, A., Schmidt, T. J., & Litwack, G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5921–5925.

Munck, A., & Brinck-Johnson, T. (1968) *J. Biol. Chem. 243*, 5556–5565.

Porzio, M. A., & Pearson, A. M. (1977) *Biochim. Biophys. Acta 490*, 27–34.

Raymoure, W. J., McNaught, R. W., & Smith, R. G. (1985) *Nature 314*, 745–747.

Raymoure, W. J., McNaught, R. W., Green, G. L., & Smith, R. G. (1986) *J. Biol. Chem. 261*, 17018–17025.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.

Smith, R. G., & Taylor, R. N. (1981) *J. Steroid Biochem. 15*, 321–328.

Taylor, R. N., & Smith, R. G. (1982) *Biochemistry 21*, 1781–1787.

Taylor, R. N., & Smith, R. G. (1985) *Biochemistry 24*, 331–343.

Taylor, R. N., Swaneck, G. E., & Smith, R. G. (1980) *Biochem. J. 192*, 385–303.