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ISOLATION OF UNTRANSFORMED BOVINE ESTROGEN RECEPTOR WITHOUT MOLYBDATE STABILIZATION

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Summary—A low concentration estrogen-derivatized affinity resin has been used in a rapid, single step purification of the untransformed estrogen receptor from calf uterine cytosols prepared without sodium molybdate. The procedure isolates the M_r 65,000 estrogen receptor in association with the bovine heat shock protein hsp90. Small amounts of proteolyzed receptor ranging in size from M_r 50,000 to 60,000 are also present in the purified extracts. Results from affinity chromatography of receptor cytosols either untreated or presaturated with estradiol suggest that two proteins of M_r 22,000 and 38,000 are co-purified with the untransformed receptor complex and may represent additional nonhormone-binding components of the native receptor form. Some indication of the stability of protein–protein interactions within the oligomeric complex has been derived from differential salt elution studies with heparin–sepharose and affinity gel-immobilized untransformed receptor. On size exclusion high performance liquid chromatography the untransformed complex eluted with a Stokes radius of 75 ± 2 Å ($n = 18$), but was shown to be sensitive to extended ultracentrifugal analysis dissociating to the receptor homodimer, sedimentation coefficient 5.3 ± 0.3 *s* ($n = 5$). Preliminary data on urea- and heat-induced transformation of the isolated receptor to the DNA-binding state is presented.

INTRODUCTION

Our laboratory has reported the purification by affinity chromatography of untransformed, molybdate-stabilized estrogen receptor from calf uterus [1, 2]. This method required cellulose–phosphate and heparin–sepharose chromatography as initial steps in the purification and isolated the M_r 65,000 estrogen receptor associated noncovalently with bovine heat shock protein hsp90 [2]. Both protein constituents have been chemically characterized by peptide sequence analysis [3–5]. The nonsteroid binding hsp90 has been identified as a common component of all untransformed, molybdate-stabilized receptors [6–8].

In this paper we describe a rapid, single step procedure for isolating untransformed estrogen receptor of high purity without molybdate stabilization. Our results indicate a component structure for the isolated complex consisting of the receptor subunit and hsp90. Two other proteins may also be associated with the complex. The purified, untransformed receptor has been characterized by size-exclusion HPLC and sedimentation analysis in hypotonic buffers. Preliminary data on urea and heat-induced transformation of the isolated receptor to the DNA-binding state is presented.

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Common names and abbreviations: PGD buffer, 10 mM potassium dihydrogen orthophosphate pH 7.4 containing 10% (v/v) glycerol and 1 mM dithiothreitol; TED buffer, 10 mM Tris (hydroxymethyl)methylamine pH 7.4 containing 1 mM EDTA and 1 mM dithiothreitol; HPLC, high-performance liquid chromatography; tamoxifen aziridine, (z)-1-[4-(2-[*N*-aziridinyl]ethoxy)-phenyl]-1,2-diphenyl-l-butene; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; M_r, relative molecular mass; 90K, 65K, etc. proteins, proteins with M_r = 90,000, 65,000, etc; hsp90, heat shock protein of M_r 90,000.

MATERIALS AND METHODS

Materials

Estradiol-17β and double-stranded calf thymus DNA were purchased from Sigma, [2,4,6,7-^{3}H]-estradiol-17β (83 Ci/mmol) and EN^{3}HANCE from New England Nuclear, [^{3}H]tamoxifen aziridine (26 Ci/mmol) from Amersham, hydroxyapatite (Bio-gel HTP) from Bio-Rad, Sephadex LH-20 from Pharmacia. Standard proteins myoglobin, ovalbumin, bovine serum albumin, catalase (bovine liver) and ferritin were obtained from Serva and thyroglobulin (bovine thyroid) from Pharmacia. Urea (ultra pure grade) was a Schwarz/Mann product.

Buffers

PGD buffer: 10 mM potassium dihydrogen orthophosphate pH 7.4 at 22°C containing 10% (v/v) glycerol and 1 mM dithiothreitol. TED buffer: 10 mM Tris(hydroxymethyl)methylamine pH 7.4 at

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22°C containing 1 mM EDTA and 1 mM dithiothreitol. These buffers were stored at 4°C. PGD buffers containing urea were freshly prepared for each experiment to prevent cyanate formation [9].

Purification of estrogen receptor

Calf uterine tissue (200 g) was homogenized in 2.7 vol of PGD buffer and the cytosol fraction was stirred for 1–2 h with 30 ml of estrogen-linked affinity resin. The suspension was poured into a scintered funnel and the gel was washed with PGD buffer (2 × 200 ml). After transfer to a glass C26/40 column (40 cm long × 26 mm i.d., Pharmacia) the gel was thoroughly washed by resuspension in PGD buffer (4 × 200 ml) and allowing the buffer to drain. The washed gel was transferred to a measuring cylinder with PGD buffer (50 ml) and the suspension was divided equally among 8 plastic centrifuge tubes. Estradiol in ethanol was added to 5 μM concentration and after a 1 h exchange reaction at 4°C affinity gel-bound receptor was recovered by centrifugation and filtration of the supernatant. A 25 ml wash with buffer containing no estradiol completed removal of the eluted receptor from the affinity gel and the combined receptor extract (75 ml) was filtered through a small column of heparin–sepharose (1 ml). After elution of heparin–sepharose-immobilized receptor with PGD buffer containing 0.8 M KCl (2.5 ml) the concentrated receptor extract was dialyzed against 50 mM NH_4HCO_3 containing 0.1% SDS and lyophilized.

Receptor recovered after affinity chromatography or after concentration with heparin–sepharose was estimated by an exchange assay [1] after adsorption of the steroid–receptor complex to hydroxyapatite. For sedimentation analysis and size-exclusion HPLC receptor was eluted from the affinity gel with 0.3 μM [^{3}H]estradiol (93 Ci/mmol) and for fluorography experiments 50 nM [^{3}H]tamoxifen aziridine (26 Ci/mmol) was used for receptor recovery.

Analytical SDS–PAGE

Slab gels with 15% (w/v) acrylamide were prepared for analytical SDS–PAGE as previously described [1]. Stacking gels contained 4% acrylamide and bisacrylamide was used as crosslinker in all gels. Electrophoretic separations were performed according to Laemmli[10] using the following standard proteins (Pharmacia) as molecular weight markers: phosphorylase b 97,400 [11], bovine serum albumin 67,000 [12], ovalbumin 43,000 [13], carbonic anhydrase 29,000 [14], soybean trypsin inhibitor 20,100 [15] and α-lactalbumin 14,400 [16]. Proteins were detected by silver staining [16]. Lanes of interest in these gels were scanned at 550 nm in a Varian Techtron 635 spectrophotometer.

After electrophoresis slab gels were prepared for fluorography by staining with 0.5% (w/v) Coomassie brilliant blue R-250 in 10% (v/v) acetic acid/30% (v/v) isopropyl alcohol for 30 min. The gels were destained overnight in 5% (v/v) acetic acid 16.5% (v/v) methanol, impregnated with EN^{3}HANCE according to manufacturer recommendations and dried on a Bio-Rad slab gel drier. Fluorographs were obtained using Kodak X-Omat film exposed at −70°C.

Ultracentrifugal analysis

After removal of unbound radiolabeled hormone by Sephadex LH-20 chromatography [1] affinity chromatography-purified receptor–[^{3}H]estradiol complexes (0.2 ml aliquots) were layered on 4.75 ml glycerol gradients (16–36% v/v glycerol) prepared in PGD buffer, with and without 20 mM sodium molybdate. Gradients were centrifuged over 17 h in a Beckman L8-70 ultracentrifuge at 237,000 *g* and 4°C for an accumulated $\omega^2 t$ (ω = angular velocity in radians per sec) of 1.66×10^{12} radians^2 sec^{-1} using an SW55 rotor. Fractions (0.1 ml) were collected by piercing the bottom of the tube and radioactivity was measured by scintillation counting in a LS 5800 Beckman liquid scintillation spectrometer. Standard proteins used as external markers were catalase, sedimentation coefficient ($s_{20,\omega}$) 11.3 *s* [17], and bovine serum albumin, 4.4 *s* [18]. Internal markers human [^{14}C]γ-globulin, 6.6 *s* [19] and [^{14}C]ovalbumin, 3.5 *s* [19] were detected by scintillation counting.

Analytical size-exclusion HPLC

Affinity chromatography-purified receptor–[^{3}H]-estradiol complexes were prepared for analytical size-exclusion HPLC by removing free radiolabeled steroid with Sephadex LH-20. Chromatography was carried out on a Varian Model 5000 liquid chromatograph fitted with a TSK-4000 SW exclusion column (Beckman, 7.5 × 300 mm) and a 10 cm guard column. Membrane filtration was used to remove particulate material from all buffers (Alltech, 0.45 μm nylon membranes) and samples (Millex-HV$_4$ 0.45 μm filters). The column was jacketed with cooling water (4°C) and equilibrated with PGD buffer with or without 20 mM sodium molybdate or 3 M urea as indicated. Samples were applied with a syringe-loaded injector (Valco) fitted with a 0.2 ml loop. A flow rate of 1 ml/min was maintained during elution and fractions collected at 0.2 min intervals were measured for tritium content.

Column void volumes (V_0) were determined by exclusion of calf thymus DNA. For each equilibration buffer the column was calibrated with myoglobin, Stokes radius (R_s) 20.1 Å [20], ovalbumin, R_s 28.6 Å [20], bovine serum albumin, R_s 61.5 Å [20] and thyroglobulin, R_s 86 Å [21]. The total liquid volume (V_t) of the column was determined with [^{3}H]estradiol. Standard curves of $(K_d)^{1/3}$ vs R_s were plotted according to Porath[22] where $K_d = V_e - V_0)/(V_t - V_0)$. V_0 is the elution volume of the protein studied.

Interaction of purified receptor with calf thymus DNA

The above size-exclusion HPLC system was used for receptor–DNA interaction studies. Calf thymus DNA in PGD buffer (2 mg/ml) was submitted to preparative exclusion chromatography and only the void volume fractions were combined for incubation with affinity chromatography-purified receptor. After removal of unbound radiolabeled hormone with Sephadex LH-20 extracts containing purified receptor–[^{3}H]estradiol complexes in PGD buffer were mixed 1:1 with buffer (control) or with aliquots containing the isolated DNA. The control sample was immediately subjected to size-exclusion HPLC and after a 30 min incubation period at 4°C the receptor–DNA mixture was analyzed in the same way. Receptor associated with DNA was eluted in the void volume of the column and the extent of receptor–DNA interaction was quantitated after a comparison of HPLC profiles obtained for receptor in the presence and absence of DNA.

A similar protocol was used to assess the influence of 20 mM sodium molybdate and 3 M urea on the DNA-binding properties of purified receptor–[^{3}H]-estradiol complexes. PGD-buffered receptor extracts were initially equilibrated in molybdate and urea buffers by dilution and were then exposed to DNA prepared in the corresponding buffer.

Radioactivity measurements

Radioactivity was measured in a LS 5800 Beckman liquid scintillation counter. Samples were counted in Beckman Ready Value scintillation fluid (3 ml). Quench correction was automatic after external standardization using the H-number method.

RESULTS

The untransformed, 9–10 *s* estrogen receptor from calf uterus was purified using a rapid, single step procedure involving affinity chromatography of cytosol prepared in hypotonic buffers free of sodium molybdate. The affinity resin utilized for receptor isolation has been previously described [1, 2] and is characterized by low nonspecific interaction properties allowing the easy removal of contaminating cytosolic proteins without urea or high salt buffers. Incubation of the affinity gel with PGD-buffered cytosol for 1–2 h at 4°C was followed by a rapid wash procedure with cytosol buffer. Figure 1 shows that maximal recovery of affinity gel-bound receptor was achieved by estradiol exchange at 4°C after a 15 min exposure to 5 μM estradiol in PGD buffer. As measured by a hydroxyapatite exchange assay using [^{3}H]estradiol the level of exchangeable, receptor-bound hormone in the eluted extract was maintained during the recovery process for at least 2 h, but was shown to substantially decrease on prolonged (24 h) mixing of the affinity gel with eluting buffer (Fig. 1). Receptor was routinely recovered over a 1 h elution

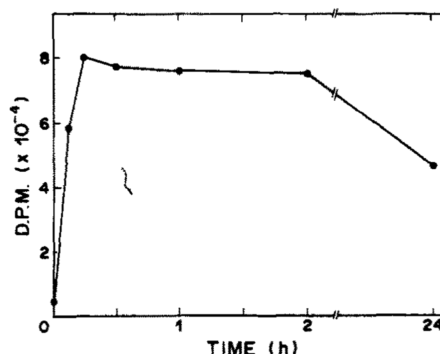

Fig. 1. Time-dependent elution of estradiol receptor from the affinity gel with estradiol. Affinity resin (3 ml) was incubated with receptor cytosol as described in the Methods section and contaminating proteins were removed by repeated washing with PGD buffer. The gel was transferred to a plastic tube with buffer (5 ml) and a 0.5 ml aliquot of the resulting suspension was withdrawn for control purposes. The suspension was made 5 μM to estradiol and was mixed by rotation. Aliquots (0.5 ml) were withdrawn at 7.5, 15 and 30 min and at 1, 2 and 24 h. Supernatants were separated from affinity gel by centrifugation and filtration and the estradiol binding capacity of each was measured by a hydroxyapatite exchange assay [1].

period. Using 30 ml of affinity resin an average receptor yield of $10.0 \pm 2.2\ \mu g$ ($n = 6$) was obtained from 200 g of uterine tissue. Similar results were obtained by using TED buffer throughout purification.

We used SDS–PAGE under denaturing conditions to characterize extracts recovered from affinity chromatography of cytosol which was either untreated or had been presaturated with estradiol. As previously observed [2] analysis of silver stained SDS–PAGE gels showed clearly that the 65K estrogen receptor, its 50K proteolytic fragment and the receptor-associated hsp90 had been selectively eliminated by the estradiol treatment (Fig. 2A). Two additional proteins with M_r 38,000 and 22,000 were also shown to be estradiol sensitive (Fig. 2A). Densitometric scanning of the stained gel indicated that the bands attributed to estrogen receptor constituted some 15% of the total protein. On this basis the recovered receptor extracts were consistently of 5–15% purity.

Highly purified estrogen receptor, covalently labeled with [^{3}H]tamoxifen aziridine, was recovered from affinity chromatography by substituting the affinity labeling reagent for estradiol. Analytical SDS–PAGE of the extract followed by Coomassie blue staining showed dominant bands attributed to the 65K receptor and hsp90 proteins (Fig. 2B, left lane). A strong band was also noted for the 38K protein. Fluorography of the gel revealed the 65K receptor as the major labeled component (Fig. 2B right lane). Consistent with our earlier observations [2], bands of radioactivity corresponding to M_r 60,000, 53,000 and 50,000 receptor fragments were also present (Fig. 2B, right lane). There was no evidence for covalent labeling of the 38K protein or the previously observed 22K unit (Fig. 2A).

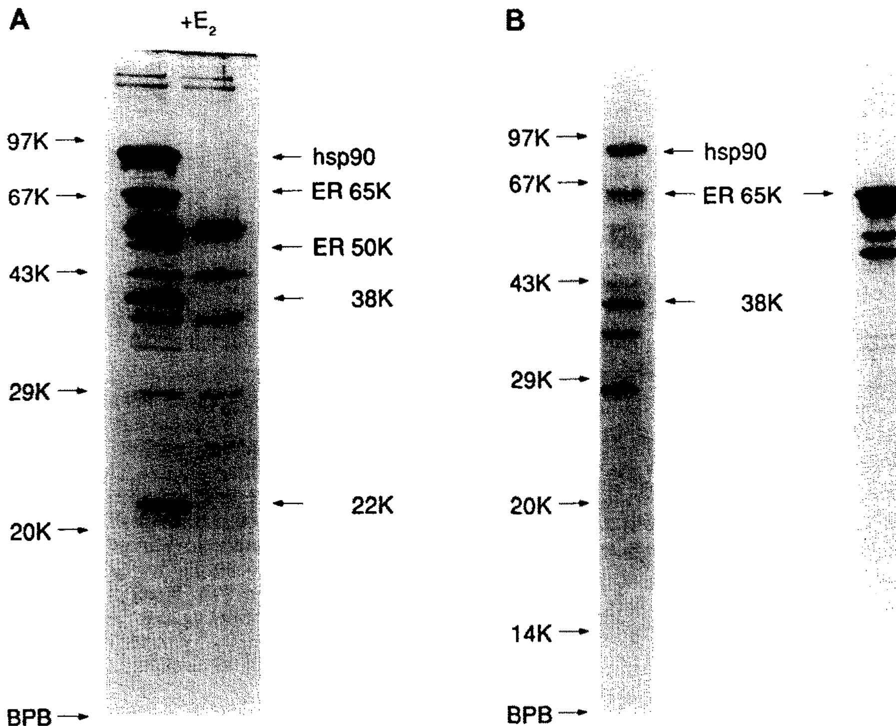

Fig. 2. Analytical SDS–PAGE of protein recovered from affinity chromatography of estradiol treated and untreated cytosol (A) and fluorography of affinity chromatography-purified, [^{3}H]tamoxifen aziridine-labeled estrogen receptor (B). (A) Receptor cytosol was divided into 2 equal portions one of which was exposed to 5 μM estradiol (+E_2) at 4°C. Both extracts were subjected to affinity chromatography and material eluted from the affinity gel with estradiol was analyzed by SDS–PAGE on a 15% (w/v) polyacrylamide slab gel. (B) Affinity labeled receptor recovered after single step affinity chromatography was electrophoresed through a 15% (w/v) polyacrylamide slab gel. After Coomassie blue staining to visualize the separated proteins (left lane) the gel was prepared for fluorography (right lane). Standard proteins used as molecular weight markers were: phosphorylase b 97K, bovine serum albumin 67K, ovalbumin 43K, carbonic anhydrase 29K, soybean trypsin inhibitor 20K, α-lactalbumin 14K, BPB-bromophenol blue.

Receptor recovered recovered from affinity chromatography with [^{3}H] estradiol was shown by analytical size exclusion HPLC in hypotonic PGD buffer to have a Stokes radius, $R_s = 75 \pm 2$ Å ($n = 18$). This was identical to that previously observed by our group for molybdate-stabilized receptor isolated from calf uterine cytosols [1]. Figure 3A shows a typical HPLC profile for the purified [^{3}H]-estradiol–receptor complex. Ultracentrifugal analysis of the same preparation, in PGD-buffered glycerol gradients, unexpectedly gave a low sedimentation coefficient (5.3 ± 0.3 *s*) ($n = 5$) (Fig. 3C). The result suggested that the untransformed receptor was unstable to extended (17 h) centrifugation through glycerol gradients and under these conditions dissociated to the smaller 5 *s* dimeric form. These sedimentation and HPLC elution properties were examined in parallel with those of the purified receptor extract equilibrated by dilution in buffer containing 20 mM sodium molybdate. A sharp, symmetrical peak of radioactivity was obtained for the [^{3}H]-estradiol–receptor complex during size exclusion HPLC in molybdate buffer (Fig. 3B). The receptor elution volume corresponded to a Stokes radius, $R_s = 73 \pm 1$ Å ($n = 3$). In the presence of molybdate the purified, untransformed receptor was stabilized against dissociation during ultracentrifugal analysis and sedimented at 8.8 ± 0.3 *s* ($n = 2$)—a value closely comparable with those previously reported [1, 23]. These observations confirmed the presence of all the protein components which constitute the untransformed receptor.

We considered the possibility that the isolation of the estrogen sensitive 38 and 22K proteins might have resulted from their noncovalent association with interaction sites available on the receptor subunit and/or the hsp90 component of the untransformed receptor complex. The affinity between such contact domains might allow the copurification of all four proteins during affinity chromatography. Some insight into the stability of these associations was derived from differential salt elution studies carried out with the purified receptor immobilized either on heparin–sepharose or on the affinity resin prior to recovery with estradiol. Figure 4A shows that buffer containing 0.12 M KCl readily eluted the hsp90 and 38K proteins from heparin–sepharose containing immobilized, affinity chromatography purified receptor.

Estradiol binding activity was subsequently recovered from the gel with buffer containing 0.8 M KCl (not shown). A parallel experiment using salt-free wash buffer indicated a gradual leaching of hsp90 from the heparin–sepharose gel (Fig. 4B). Estrogen receptor and the 38K protein were found only in the 0.8 M KCl eluate together with residual amounts of hsp90 (Fig. 4B). In this experiment the elution properties of the 22K protein species were not assessed since levels were too low for positive identification by analytical SDS–PAGE.

In differential salt elution studies with affinity gel-bound receptor significant differences were noted in the elution properties of hsp90 and the 38K protein when compared to those described with heparin–sepharose. After an initial salt-free buffer wash the affinity resin was treated with buffer containing increasing salt concentrations ranging from 0.10 to 0.18 M KCl (Fig. 5). Although hsp90 and the 22K component were present in the initial buffer wash, release of the 38K protein was only induced with 0.1 M KCl (Fig. 5). In contrast to the experiment with heparin–sepharose a gradual rather than a sudden release of these three proteins was observed with further increases in salt concentration (Figs 4 and 5). Residual amounts of hsp90 and the 38K protein were

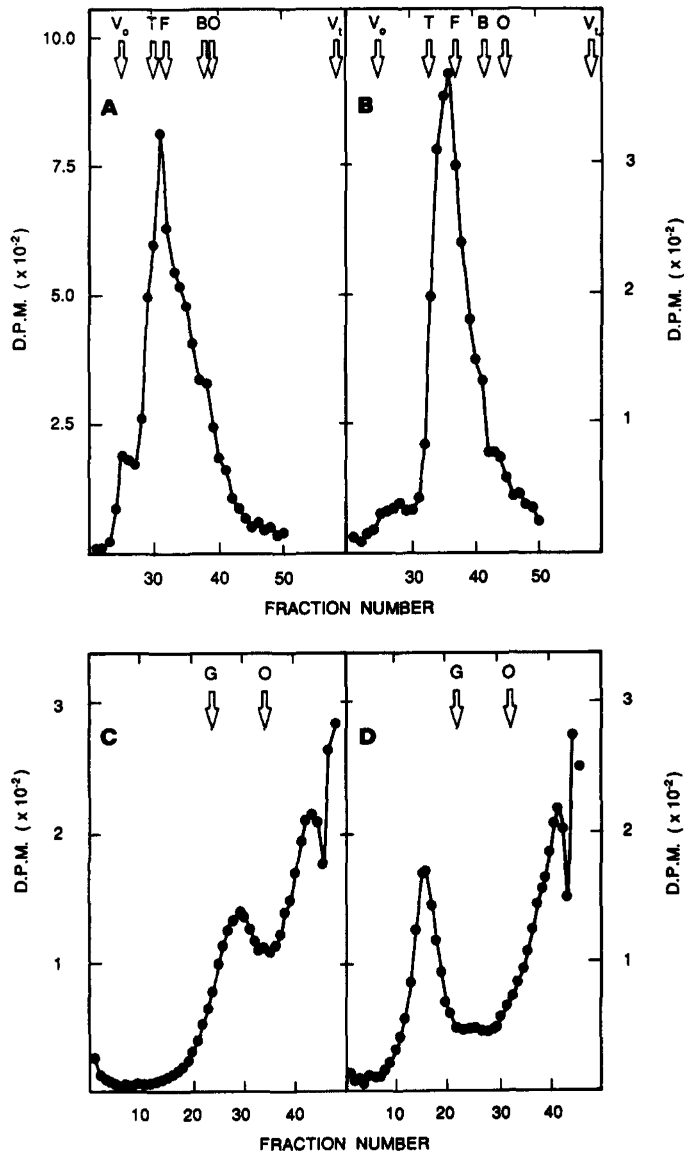

Fig. 3. Size exclusion HPLC and sedimentation analysis of affinity chromatography-purified, untransformed receptor in the presence and absence of sodium molybdate. An extract of purified, [^{3}H]estradiol-labeled receptor in PGD buffer was equilibrated by 1:1 dilution either with PGD buffer or with buffer containing 40 mM sodium molybdate. Both preparations were then subjected to size exclusion HPLC (A, B) and ultracentrifugal analysis (C, D) in the corresponding buffers. (A, C) Analyses were carried out in PGD buffer, (B, D) in PGD buffer containing 20 mM molybdate. Calibration standards are as described in Materials and Methods.

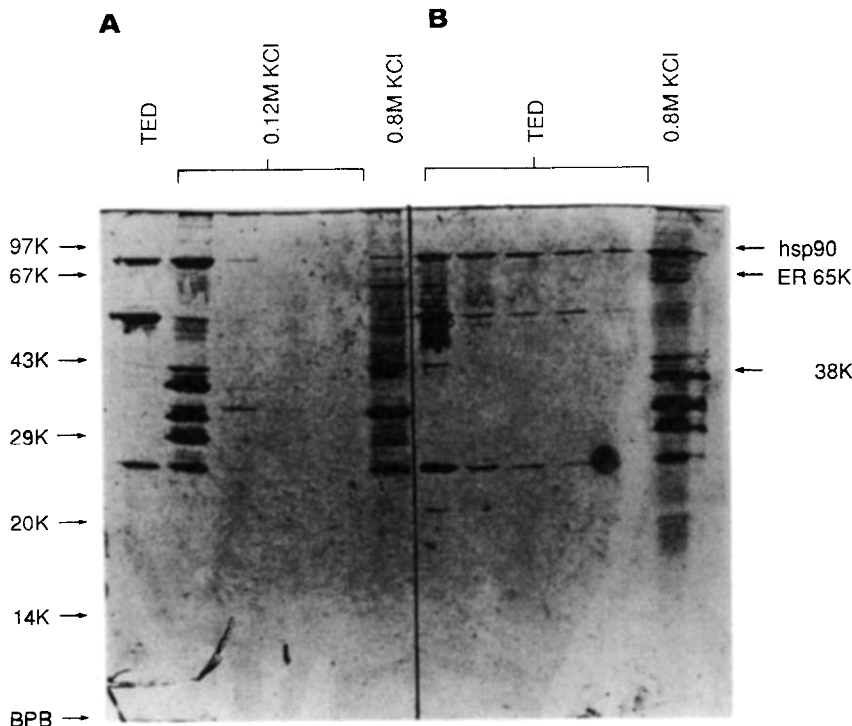

Fig. 4. The salt elution properties of the protein components of purified, untransformed estrogen receptor immobilized on heparin–sepharose. (A) An estrogen receptor preparation (5 ml), purified by affinity chromatography in TED buffer, was applied to a 0.25 ml heparin–sepharose column over 1 h at 4°C. The column was washed sequentially with 1 ml of TED buffer, four 1 ml aliquots of TED buffer containing 0.12 M KCl and finally with 0.8 M KCl TED buffer. The wash fractions were dialyzed against 50 mM NH_4HCO_3 buffer containing 0.1% SDS and lyophilized. After electrophoresis on a 15% (w/v) SDS–polyacrylamide slab gel the separated proteins were visualized by silver staining. (B) In a parallel experiment five consecutive 1 ml TED buffer washes were applied to the gel before elution with 0.8 M KCl. Molecular weight standards were as for Fig. 2.

still evident on recovery of estrogen receptor with estradiol (Fig. 5, lane 7). In a parallel experiment the affinity resin was washed repeatedly with salt-free buffer. Decreasing levels of hsp90 and the 22K unit were observed in successive wash fractions (data not shown). Most of the hsp90 protein however was recovered together with the estrogen receptor and the 38K component on exposure of the affinity gel to estradiol (Fig. 5, lane 8). This same extract also contained residual amounts of the 22K protein (Fig. 5, lane 8).

Size exclusion HPLC was utilized to study the interaction properties of the affinity chromatography-purified receptor with double-stranded calf thymus DNA. Solubilized DNA was fractionated by preparative size exclusion HPLC in PGD buffer and only the DNA eluted in void volume fractions was used for receptor studies. We noted a marked change in the size exclusion HPLC elution profile of purified, [^{3}H]estradiol-labeled receptor following incubation with DNA. Figure 6A shows that after allowing for a small degree of receptor aggregation approximately 28% of the receptor recovered during chromatography was eluted in the void volume in association with DNA. In a control experiment affinity chromatography-purified receptor not exposed to DNA gave an elution profile characteristic of the untransformed receptor complex (Fig. 6A, see also Fig. 3A). This same control elution pattern was repeated for six affinity chromatography-purified receptor preparations. The DNA binding of receptor in these extracts however was highly variable and ranged from zero to 40% ($x = 19.1 \pm 14.2\%$ ($n = 6$)). Addition of molybdate to the purified receptor extract prior to incubation with DNA precluded receptor-DNA interaction. The elution profile of molybdate-stabilized receptor was unaltered by exposure to DNA (data not shown).

The influence of 3 M urea on the size exclusion HPLC profile and the DNA binding capacity of purified receptor was also studied. After equilibration in PGD buffer containing urea the receptor preparation described in Fig. 6A was chromatographed with and without prior incubation with DNA. Figure 6B shows that in the absence of DNA urea-treated receptor was eluted in a broad peak ($R_s = 59$ Å) between the elution volumes for the standard proteins ferritin and bovine serum albumin. Substantial aggregation of the receptor was also noted. In the presence of DNA urea-equilibrated receptor eluted at the void volume as a single, sharp peak (Fig. 6B). Assuming that the level of receptor aggregation remained

unchanged on exposure to DNA this DNA-bound receptor fraction represented 47% of recovered hormone binding activity and showed that a considerable increase in the DNA binding capacity of urea-treated over untreated receptor (cf. Fig. 6A) had been achieved.

The above results indicated successful urea-induced transformation of highly purified receptor to the DNA-binding form. However, because of the extensive receptor aggregation observed with urea treatment in these and subsequent experiments alternative methods of receptor transformation were investigated. Although heating the purified receptor extract at 25°C for 30 min achieved some conversion of receptor to the transformed state receptor aggregation was still a significant feature (not shown). Severe losses of receptor-bound [^{3}H]estradiol were also evident indicating a sensitivity of the receptor-hormone complex to the elevated temperature conditions used. A reduction in incubation temperature to 15°C was beneficial however. The results of a time study on receptor transformation carried out at this temperature are shown in Fig. 7. A gradual and complete conversion of untransformed to transformed receptor (R_s = 54 Å (n = 3)) over a 20 min period is clearly demonstrated with little evidence of receptor aggregation (Fig. 7).

DISCUSSION

This report describes a rapid, affinity chromatography-based method for isolating untransformed estrogen receptor directly from calf uterine cytosol prepared in molybdate-free buffers. Hitherto, highly purified untransformed steroid receptors have been

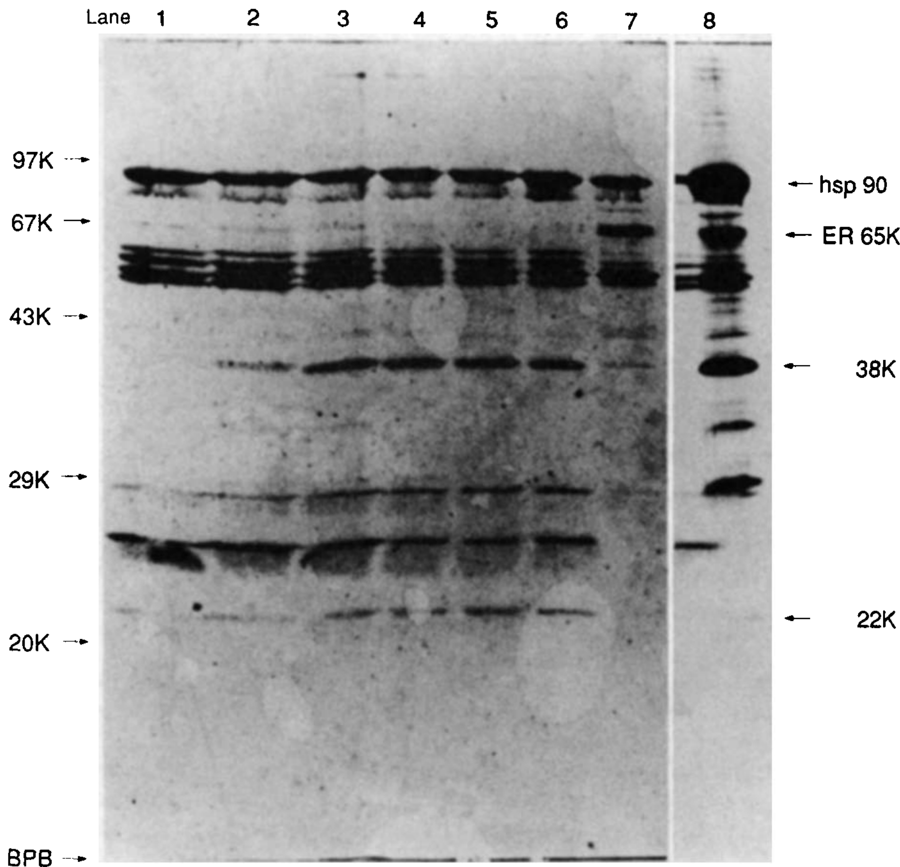

Fig. 5. Differential salt elution of protein components from affinity resin-immobilized untransformed estrogen receptor. After incubation with receptor cytosol affinity resin (4 ml) was extensively washed with TED buffer and divided equally between two plastic tubes. A 5 ml aliquot of TED buffer was added to both tubes and after mixing and centrifugation the supernatants were recovered by filtration through a nylon disc. Using the same procedure the gel in one tube was washed successively with 5 ml aliquots of TED buffer containing increasing KCl concentrations. A final wash with TED buffer (5 ml) containing 5 μM estradiol was used to recover receptor together with other retained proteins. All recovered supernatants were dialyzed against 50 mM NH_4HCO_3 buffer containing 0.1% SDS and lyophilized. After electrophoresis on a 15% (w/v) SDS–polyacrylamide slab separated proteins were visualized by silver staining. Electrophoresed samples were: lane 1, TED wash, lanes 2–6, washes with TED buffer containing 0.10, 0.12, 0.14, 0.16 and 0.18 M KCl, respectively, lane 7, estradiol-recovered eluate. Lane 8 contains the estradiol-recovered sample from the control gel which had been washed consecutively six times with TED buffer only. Protein standards are as described in Fig. 2.

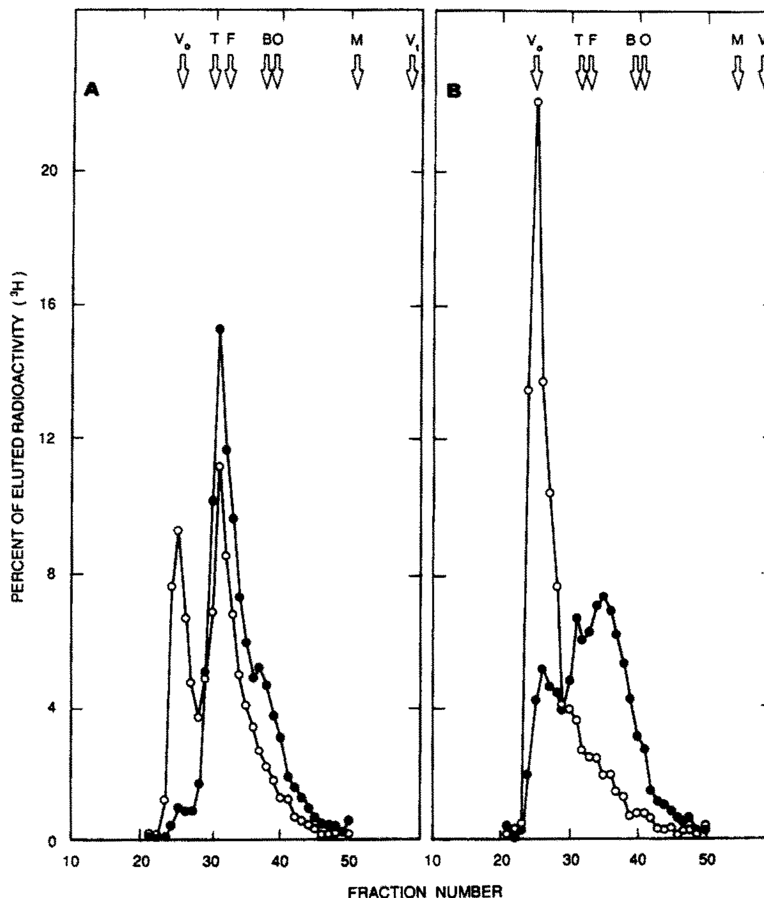

Fig. 6. DNA-binding properties of purified, untransformed estrogen receptor before and after urea-induced transformation. Calf thymus DNA was fractionated by size-exclusion HPLC and the void volume fractions were combined for receptor binding studies. (A) Purified, untransformed receptor was recovered from affinity chromatography with [^{3}H]estradiol in PGD buffer. Unbound, radiolabeled hormone was removed by Sephadex LH-20 chromatography and the receptor extract was equilibrated by 1 : 1 dilution with PGD buffer (control) or buffer containing the fractionated DNA. The control sample (●) was immediately subjected to size-exclusion HPLC and after a 30 min incubation at 4°C the receptor–DNA mixture (○) was analyzed in the same way. (B) After Sephadex LH-20 filtration the receptor extract from A was equilibrated by dilution with 3 M urea (control, ●) and urea buffer containing DNA (○) and both samples were analyzed by size exclusion HPLC. Calibration standards are as described in Materials and Methods.

isolated only under the stabilizing influence of sodium molybdate which protects against salt-induced dissociation [1, 24–26]. A recent report by Aranyi *et al.*[27] has described the purification, in the absence of molybdate ions, of untransformed progesterone receptor covalently stabilized by chemical cross-linking.

The purification of estrogen receptor in its native form is largely attributed to the special properties of our affinity absorbant which is derivatized in low concentration with estrone carboxymethyloxime, a low affinity, receptor binding estrogen [1]. The resin is characterized by very low nonspecific interaction with contaminating proteins which are easily removed with hypotonic buffers. The rapid isolation technique minimizes receptor proteolysis and the protein is biospecifically eluted as the untransformed receptor complex. Our purification scheme contrasts with methodologies previously reported for receptor isolation without molybdate stabilization [28–30]. These alternative procedures incorporated buffers with high salt concentration, chaotropic agents and organic solvents and isolated receptor in its M_r 65,000 monomeric form.

Our isolation procedure yields untransformed receptor composed of the M_r 65,000 steroid binding unit and bovine hsp90. Steroid binding receptor fragments, varying in size from M_r 50,000 to 60,000 were also isolated, but in much smaller amounts. All of these protein species were previously observed by our group with a more elaborate purification scheme involving sequential cellulose phosphate, heparin–sepharose and affinity chromatography of molybdate-stabilized cytosol [2]. Cellulose phosphate chromatography was specifically included to limit proteolytic modification of receptor [2]. Results with

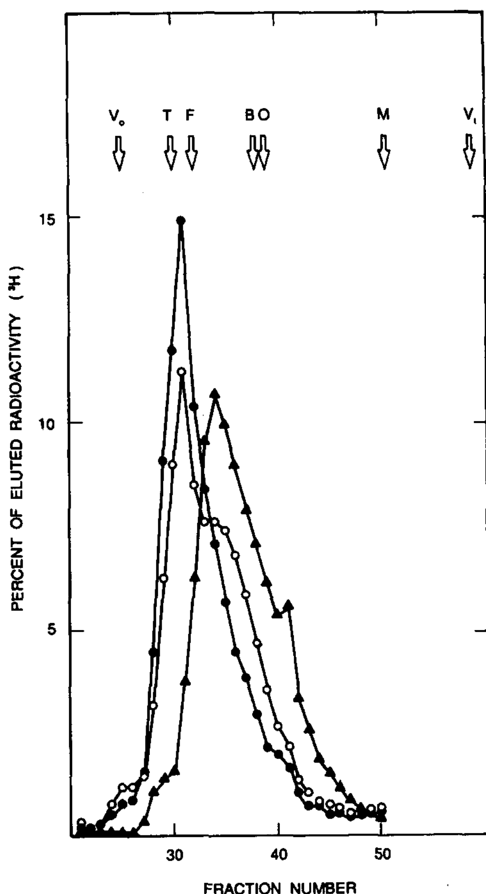

Fig. 7. Time-dependent, heat-mediated transformation of affinity chromatography-purified receptor. An affinity chromatography-purified receptor-[^{3}H]estradiol extract was filtered through Sephadex LH-20 to remove free hormone. The preparation was then incubated at 15°C and aliquots were removed for analysis by size exclusion HPLC after zero (●), 10 min (○) and 20 min (▲) time intervals. The column void volume (V_0) and total volume (V_t) as well as the elution volumes for calibration standards are as indicated: thyroglobulin (T), ferritin (F), bovine serum albumin (B), ovalbumin (O) and myoglobulin (M).

the current, more direct method showed similar low levels of receptor degradation.

The possibility exists that the M_r 22,000 and M_r 38,000 protein species recovered with receptor during affinity chromatography may represent additional nonhormone binding components of the untransformed estrogen receptor complex. Although the association of hsp90 with steroid hormone receptors is an accepted feature of untransformed receptors [31] a number of workers have alluded to additional, as yet unidentified components [23, 31, 32]. There is now strong evidence for the association of steroid receptors with RNA [33–36] and a recent report by Ali and Vedeckis [36] suggest that a 25,000 Da tRNA may be involved. Based on immunological data a 59K protein appears to be associated with hsp90 in androgen, estrogen, glucocorticoid and progesterone receptors from rabbit uterine and liver cytosol [37]. Our differential salt elution studies with untransformed receptor immobilized on heparin-sepharose or the affinity resin indicated similar elution patterns for hsp90 and the M_r 22,000 protein, but a different trend for the M_r 38,000 component. While these observations may be explained by protein–protein interactions between the M_r 22,000 component and hsp90 and the M_r 38,000 protein and the estrogen receptor, further experimental evidence is required to support such a proposal. The influence of receptor-bound steroid on the dissociation of hsp90 from receptor in these studies should also be considered. Our results indicate that the heat shock protein appears to be easily dissociated from the heparin–sepharose bound estradiol–receptor complex, but that hsp90 was more resistant to release from untransformed receptor immobilized on the affinity gel via estrone carboxymethyloxime. The binding affinity of this estrogen for receptor is approximately 10^4 times lower than that of estradiol [38] and its interaction with receptor during affinity chromatography may not achieve the same conformational changes induced by the natural hormone [39]. These alterations in receptor structural conformation are understood to favour the dissociation of the receptor–hsp90 heteromeric complex [40–42].

During extended ultracentrifugal analysis on density gradients the purified untransformed receptor dissociated to a 5.3 *s* protein consistent with sedimentation data reported for transformed, dimeric receptor [23, 43, 44]. The presence of two receptor subunits in the untransformed, calf uterine receptor is supported overwhelmingly in the literature [23, 45]. Immunological analysis of the untransformed, molybdate-stabilized receptor suggests the association of two hsp90 molecules with two steroid-binding subunits [23]. Transformation may then simply involve the release of the receptor homodimer from the native receptor form [23].

A significant, albeit highly variable proportion of the purified, untransformed receptor preparation was found to bind to calf thymus DNA, as determined by size-exclusion HPLC. This interaction was further enhanced in the presence of 3 M urea concomitant with a decrease in receptor Stokes radius from 75 and 59 Å. DNA binding was completely inhibited by sodium molybdate a result consistent with the prevention of receptor transformation for all steroid receptors [26, 46–48]. Transformation of glucocorticoid and progesterone receptors to the DNA-binding state requires the dissociation of hsp90 from receptor monomers [31, 40]. For the estrogen receptor evidence suggests that the transformation mechanism may release receptor as the homodimer (R_s ~60 Å) [23]. The results of the present study indicate a fragile association of nonhormone binding component(s) with the purified estradiol-liganded receptor in the absence of molybdate ions. The levels of DNA interaction observed with untransformed receptor extracts may have resulted from partial receptor conversion to the DNA-binding form. Our

study of urea-promoted DNA-binding by purified estrogen receptor has been complicated by a tendency for the receptor to aggregate in urea buffers. With cytosolic receptor preparations Hutchens *et al.*[49] have described an almost quantitative urea-induced binding of receptor to DNA-linked absorbants with no evidence of receptor aggregation. Our group has confirmed the observations of Hutchens *et al.* with crude receptor extracts (unpublished). The aggregation phenomenon then appears to be peculiar to highly purified receptor which may be prone to the formation of large, aggregated complexes. Indeed we have noted the gradual aggregation of purified receptor on standing at 4°C (unpublished).

The highly purified, untransformed estrogen receptor should prove to be very useful in studies aimed at defining the structural alterations which result in enhanced affinity for DNA. In particular such studies should clarify the involvement of receptor-associated proteins (e.g. hsp90) in the modulation of receptor–DNA interaction and assess hormone and anti-hormone influences on the binding of receptor to specific, target DNA sequences [50, 51]. Isolation of highly purified estrogen receptor in association with hsp90 and perhaps the M_r 22,000 and M_r 38,000 proteins allows the separation of each component by preparative SDS–PAGE followed by electroelution. The M_r 65,000 receptor monomer and hsp90 have already been isolated in this way and chemically characterized by sequence analysis [3–5]. A similar approach should provide structural information on the M_r 22,000 and M_r 38,000 protein species. We are currently using covalently cross-linked, affinity labeled estrogen receptors and denaturing PAGE to further elucidate the structure of the purified oligomeric receptor.

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