# Metal Binding Sites of the Estradiol Receptor from Calf Uterus and Their Possible Role in the Regulation of Receptor Function†

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ABSTRACT: The existence of putative metal binding sites on the estradiol receptor (ER) molecule from calf uterus was evaluated by immobilizing various divalent metals to iminodiacetate–Sepharose. ER from both crude and highly purified preparations binds to metal-containing adsorbents complexed with Zn(II), Ni(II), Co(II), and Cu(II), but not to those complexed with Fe(II) and Cd(II). Elution of ER was obtained by chelating agents or by imidazole, thus indicating that histidine residues on the ER molecule are involved in the interaction with the metal. Analysis of affinity-labeled ER by [$^3$H]tamoxifen aziridine after elution from a column of Zn(II)-charged iminodiacetate–Sepharose showed that ER fragments obtained by extensive trypsinization were also bound. Zn(II) and the same other metals able to bind ER, when immobilized on resins, inhibit the binding of estradiol to the receptor at micromolar concentrations. This inhibition is noncompetitive and can be reversed by EDTA. The inhibition of the hormone binding was still present after trypsin treatment of the cytosol, and it was abolished by preincubation with the hormone. Micromolar concentrations of these metals were able to block those chemical–physical changes occurring during the process of ER transformation in vitro. Furthermore, if added to pretransformed ER–hormone complex, they strongly inhibited the binding of the complex to isolated nuclei. The presence of metal binding sites that modulate the ER activity in the hormone binding domain of ER is therefore speculated. Since progesterone receptor showed the same pattern of binding and elution from metal-containing adsorbents, the presence of metal binding regulatory sites could be a property of all steroid receptors.

The molecular mechanism of action of the steroid hormones has not been completely elucidated (Gorski et al., 1986). During the last few years, sequencing of cDNAs coding for all steroid receptors added new insight to their structure (Hollenberg et al., 1985; Green et al., 1986; Weinberger et al., 1986; Arriza et al., 1987; Gronemeyer et al., 1987; Krust et al., 1986, Kumar et al., 1986). A common molecular architecture has been found in these molecules. There are two highly conserved regions devoted to the binding of DNA and hormone, respectively, separated by less conserved hinge regions. A third, N-terminal domain has been identified; it probably plays an important role in the initiation of RNA synthesis, though it is not well conserved. The oncogene potentiator protein v-*erb*-A encoded in the avian erythroblastosis virus has striking homology to the hormone binding and DNA binding domains of the steroid receptors; its cellular counterpart c-*erb*-A has been identified as the thyroid hormone receptor (Weinberger et al., 1986). The receptor domains are likely to cooperate in order to obtain full functional action. The first and very important cooperation occurs during the "transformation" of the receptor, i.e., a process triggered by the hormone and consisting of the acquisition, by the native receptor, of the ability to bind to specific DNA sequences—the steroid response elements (Milgrom, 1981; Schmidt & Litwack, 1982; Munck & Holbrook, 1984; Puca et al., 1984; Denis et al., 1988). Several mechanisms have been proposed to explain the molecular basis of this event. The question is whether the transformation involves enzymatic mechanisms or whether it simply represents an allosteric modification that unmasks the DNA binding domain (King, 1987; Godowski et al., 1987).

The DNA binding domain of the steroid receptors contains a cysteine-rich region able to fold into the so-called metal binding fingers similar to those present in many other DNA binding proteins (Berg, 1986). These supersecondary structures are generated by the coordination of the metal ion with four cysteine/histidine residues in each finger. These findings are in close correlation with reports asserting that steroid receptors are metalloproteins (Shyamala, 1975) and that the DNA binding activity of estradiol receptor (ER)[1] is destroyed by the chelation (Sabbah et al., 1987).

The availability of metal-chelating adsorbents allows further investigation of the metal binding properties of ER proteins (Porath & Olin, 1983). Moreover, MCAC allows purification of proteins on the basis of their affinity for metal ions. Differential elution conditions can suggest the amino acid residue(s) involved in this interaction.

Here we report some metal binding properties of ER and the effects of divalent metal ions on known functional properties of ER. Our results indicate possible correlations between

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[1] Abbreviations: ER, estradiol receptor; DEAE, diethylaminoethyl; MCAC, metal chelate affinity chromatography; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; TEMED, *N,N,N',N'*-tetramethylethylenediamine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; TCA, trichloroacetic acid; BT, breakthrough; PMSF, phenylmethanesulfonyl fluoride; DCC, dextran-coated charcoal; BSA, bovine serum albumin.

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the metal binding sites and the molecular function of the receptor.

## Experimental Procedures

[$^3$H]Estradiol (84–111 Ci/mmol) was purchased from New England Nuclear. [*ring*-$^3$H]Tamoxifen aziridine (22 Ci/mmol) was from Amersham. Nonradioactive estradiol was from Calbiochem. DEAE-cellulose (DE-52) was from Whatman. Sepharose, protein A–Sepharose, Superose-6B, and chelating Sepharose 6B were from Pharmacia. Guanidine hydrochloride, imidazole, and EDTA were from Sigma. Amplify was from Amersham. TEMED, acrylamide, and bis(acrylamide) were from Serva. SDS and low molecular weight protein standards were from Bio-Rad. All other reagents were analytical grade from Baker.

The following buffers were used: (A) 7.5 mM sodium phosphate buffer, pH 7.4 at 20 °C, containing 1 mM DTT and 10 mM $Na_2MoO_4$; (B) buffer A without $Na_2MoO_4$; (C) 7.5 mM sodium phosphate buffer, pH 7.4 at 20 °C, containing 250 mM sucrose, 25 mM KCl, 1 mM DTT, and 0.2 mM PMSF.

Frozen (−70 °C) calf uteri were used for cytosol preparation. Tissue was homogenized by an Ultraturrax homogenizer (Janke and Kunkel, TP 18/10) in 4 volumes of buffer A at 4 °C. The homogenate was centrifuged at 105000*g* for 60 min at 4 °C in a Ti 50.2 Beckman rotor with an L5–75 Beckman ultracentrifuge, and the supernatant was collected and is here referred to as cytosol. Cytosol was incubated with 5 nM [$^3$H]estradiol for 1 h at 4 °C and then treated with the pellet of an equal volume of charcoal suspension to remove the unbound hormone (Korenman, 1968). Nonspecific binding was determined by parallel incubation in the presence of a 300-fold excess of unlabeled estradiol. When indicated, ER was covalently labeled with [$^3$H]tamoxifen aziridine (20 nM final) stocked in 85% dimethylformamide and 15% ethanol, incubating the sample for 2 h at 0 °C and then removing the free ligand with DCC (Katzenellenbogen, 1983).

ER was partially purified by DEAE-cellulose chromatography. Cytosol (80 mL) was incubated at 4 °C with DEAE-cellulose (10 g wet weight) equilibrated in buffer A, containing 0.1 M KCl for 30 min. Resin was packed on a column (internal diameter 1.4 cm) and eluted with a 100-mL linear gradient from 100 to 400 mM KCl in buffer A. Fractions of 3 mL were collected, and the specific estradiol binding activity was measured.

A column (18.5 × 1.6 cm) of Superose 6B calibrated at a flow rate of 100 mL/h with proteins of known molecular weight was used in the experiments of exclusion chromatography.

Monoclonal antibodies to ER were purified by protein A–Sepharose (Moncharmont et al., 1982) and coupled to Sepharose 4B-CL activated by the cyanogen bromide method (Cuatrecasas, 1970). About 1 mg of purified antibody was covalently bound to each gram of resin. Prior to use, each column was washed with 0.4 N ammonium hydroxide (pH 11.6) and then equilibrated in buffer A containing 0.3 M KCl. In the experiments of immunoaffinity, the sample was brought to 0.3 M KCl and applied onto a 1.2-mL column of Sepharose–anti-ER antibodies. After a sequential wash with buffer A containing 0.3 M KCl (40 mL), 1 M guanidine in buffer A (30 mL), and water (7 mL), the column was eluted with 0.4 N $NH_3OH$ (pH 11.6).

In all experiments of MCAC, resins of iminodiacetate-substituted agarose were packed in distilled water according to Porath (Porath & Olin, 1983). Each column was then loaded with the appropriate metal ion by applying 3 bed volumes of a 5 mg/mL solution of the metal in $H_2O$ as chloride. Chelating Sepharose 6B has a total capacity for $ZnCl_2$ of about 30 μmol/mL of swollen gel. Finally, columns were equilibrated in buffer A containing 0.5 M KCl.

Even though the solid-phase complexation chemistry is slightly different from that in solution, the preference given to MCAC was due to the possible use of this chromatographic technique as a tool in ER purification. Iminodiacetate-substituted agarose was chosen among the chelator gels, because in this gel the bound metal ions have a greater number of free coordination sites. In this manner, chelate structures involving more than two ligands on the protein can be formed. The iminodiacetate–chelone offers very low steric hindrance to proximal interactions; it is likely that supersecondary structures, such as the metal binding "fingers", also could bind to the resin.

Sucrose gradient analysis as described previously (Puca et al., 1971) was performed in buffer A.

For electrophoretic analysis, samples were concentrated to a final volume of 100 μL by precipitation with 10% TCA for 30 min at 0 °C. After centrifugation at 6000*g*, pellets were washed twice with ether–ethanol (1:1), resuspended in a buffer containing 62.5 mM Tris-HCl, pH 6.8, 0.1% SDS, 10% glycerol, and 50 mM DTT, and boiled for 2 min. SDS–PAGE electrophoresis was then performed according to Laemmli (1970). Gels were treated with Amplify, dried, and exposed to Hyperfilm, following manufacturer's instructions (Amersham).

Isolation nuclei were prepared from the crude nuclear pellet of calf uterus in buffer C. DNA concentration was determined (Giles & Myers, 1965) and adjusted to 1.2 mg/mL with the buffer. Binding of the ER–hormone complex to nuclei (0.1 mL, 120 μg of DNA) was assayed in duplicate. The binding of the complex was time, temperature, and estradiol dependent (Puca et al., 1984). Nonspecific binding, obtained by incubating unheated hormone–receptor complex with nuclei, was subtracted from values obtained. At the end of the incubation, usually 60 min at 0–4 °C, the nuclei were pelleted in a swinging-bucket rotor at 3000*g* for 10 min and washed 3 times with 4 mL of buffer B. The pellet was resuspended in 1 mL of the same buffer for measurement of radioactivity.

Radioactivity was measured by addition of 12 mL of scintillation mix (Dyna-gel from Baker) with a LS-5000 T liquid scintillator from Beckman (efficiency about 40°).

Protein concentration was measured by the Coomassie blue method using BSA as standard (Bradford, 1976).

## Results

*Metal Chelate Affinity Chromatography.* In our work, only divalent metal ions were considered: this was desirable because Zn(II) was the most probable candidate to reach the appropriate functional conformation of ER domains (Krust et al., 1986; Kumar et al., 1986). A preliminary experiment was performed by applying small volumes of cytosol, preincubated with the hormone, onto iminodiacetate–Sepharose columns precharged with various metal ions.

A buffer including 0.5 M KCl was chosen to avoid charge-controlled attraction forces (see below). It should be noted that the inclusion of 0.5 M KCl in a buffer containing 10 mM molybdate at 4 °C does not modify the hormone binding capacity nor the chemicophysical characteristics of the ER. Binding of the ER complex to the gels charged with Zn(II), Co(II), Ni(II), and Cu(II) was observed; on the contrary, the ER complex passed almost completely through uncharged or Fe(II)- and Cd(II)-containing adsorbents (Table

Table I: Binding of ER to Iminodiacetate–Sepharose Columns Charged with Metal Ions[a]

| | cpm passed through | % |
|---|---|---|
| no metals | 226391 | 96 |
| Zn(II) | 78056 | 33 |
| Co(II) | 29680 | 12 |
| Cu(II) | 4874 | 2 |
| Ni(II) | 50386 | 21 |
| Fe(II) | 228310 | 97 |
| Cd(II) | 220837 | 94 |

[a] Cytosol prelabeled with [$^3$H]estradiol (1 mL, 236000 cpm) and added with 0.5 M KCl was applied onto iminodiacetate–Sepharose columns (1-mL gel bed) precharged with various metal ions. Percent refers to cpm specifically applied to the columns.

I). The quantitative differences among the adsorbents charged with Zn(II), Ni(II), and Co(II) were not repeatable, probably reflecting differences in flow rate or charging conditions. Only a very small fraction of the specific ER complex flowed through the Cu(II)-containing adsorbent. However, when a chelating agent (EDTA, 50 mM) was included in the washing buffer, we obtained a complete elution of the macromolecular bound radioactivity from the Zn(II), Ni(II), and Co(II) adsorbents, whereas the elution from Cu(II) columns was poor. In the latter, the recovery of the hormone–receptor complex was less than 30%, as opposed to 90% obtained from Zn(II), Ni(II), and Co(II), indicating that the copper metal irreversibly inactivates the hormone binding site.

To further characterize the ER binding to these resins, loading conditions were varied according to the findings of Porath on the properties of the interaction between proteins and immobilized metal ions (Porath & Olin, 1983). Among the conditions tested, the ionic strength and pH were considered interesting.

ER binding to Zn(II) columns was determined at various salt concentrations. Increasing ionic strength promoted the adsorption capacity of the complex to the resin (Figure 1A). This was due to the contrasting action of two adsorption factors. At low ionic strength, ion exchange or ionic adsorption is predominant, whereas at high salt concentration, the adsorption reflects the formation of the coordinative-bonded adsorption complex. A strong pH dependence of the coordinative binding was observed. The maximum binding was obtained at pH values greater than 8 (Figure 1B). Even though the effective values of the p$K_a$ of the imidazole groups in ER molecule are not known, it is clear that an increase in the pH of the medium increases the number of nitrogen groups available for the coordinative binding.

The residues involved in the direct metal binding were identified by affinity elution in MCAC. Competitive elution from all the resins of bound ER molecules was obtained by a gradient of increasing imidazole concentration. Figure 2 shows an imidazole gradient of partially purified ER complex from a Zn(II) column. A small portion of radioactivity and a large peak of proteins were not retained by the column. In other experiments, when the ER was labeled in the presence of an excess of cold hormone, most of the nondisplaceable radioactivity was found in the flowthrough. The imidazole gradient eluted one peak of proteins, containing most of the ER–hormone complex, and a large shoulder of this peak where there is apparently a higher state of purification of the receptor protein. A similar pattern was obtained from crude cytosol preincubated with the hormone. The ER bound to Zn(II) columns even in the absence of the hormone and was eluted by imidazole with about 80% recovery (not shown). Change of ionic strength or inclusion of a nonionic detergent (CHAPS) with the aim of increasing the proportion of receptor in the

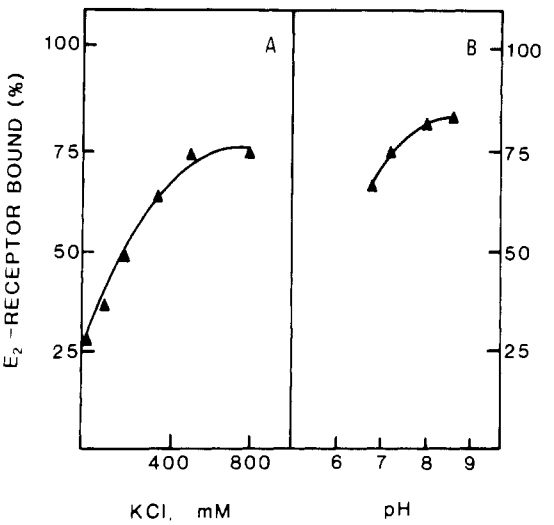


FIGURE 1: Binding of ER to iminodiacetate–Sepharose columns precharged with Zn(II). (A) Effect of ionic strength: Cytosol was preincubated with [$^3$H]estradiol in the absence or presence of an excess of cold hormone and treated with DCC to remove the free ligand. Aliquots of 250 μL (about 95000 cpm) were brought to the indicated KCl concentrations and applied onto iminodiacetate–Sepharose columns (0.6 mL) precharged with $ZnCl_2$ and equilibrated in buffer A at the same ionic strength as the sample. The specific estradiol binding capacity that passed through the columns was measured and expressed as percent of specific cpm charged. (B) Effect of pH: Aliquots of labeled cytosol (200 μL, about 65000 cpm) were diluted to 1 mL at the indicated pHs with phosphate buffer and then applied onto Zn(II)–iminodiacetate–Sepharose columns (1 mL) equilibrated in buffer containing 0.5 M KCl and at the same pH as the samples. The specific estradiol binding capacity that passed through the columns was measured and expressed as the percentage of specific cpm charged.

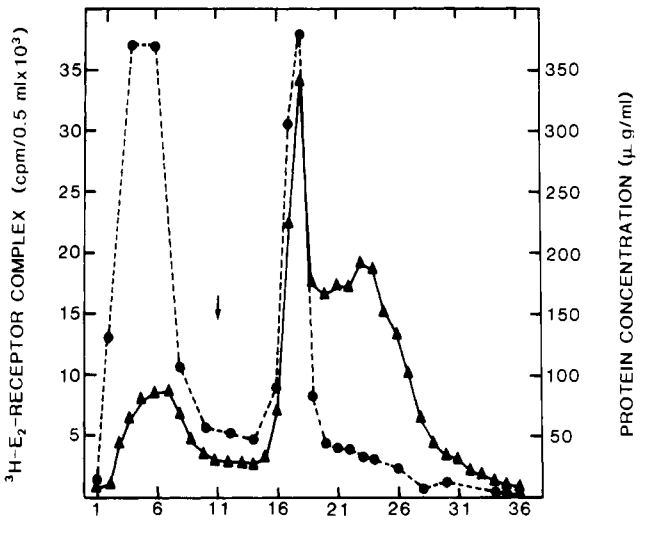


FIGURE 2: Chromatography on Zn(II) columns of partially purified ER. Cytosol preincubated with [$^3$H]estradiol was partially purified by DEAE-cellulose. Fractions containing the peak of bound estradiol were pooled and brought to 0.5 M KCl, and the pH was adjusted to 8.4. The peak was applied onto an iminodiacetate–Sepharose column (4.5 mL) precharged with $ZnCl_2$ and equilibrated in buffer A containing 0.5 M KCl (pH 8.4). ER was eluted with a linear gradient of 0–100 mM imidazole in buffer A containing 0.5 M KCl (pH 8.4). Fractions (2 mL) were collected and analyzed for radioactivity, and protein concentration was determined. The arrow indicates application of the gradient. (▲) cpm total; (●) protein concentration.

higher state of purity did not modify the elution profile. Sucrose gradient analysis of the peak associated with the OD revealed the presence of aggregates of ER molecules that sedimented at the bottom of the gradients; these aggregates

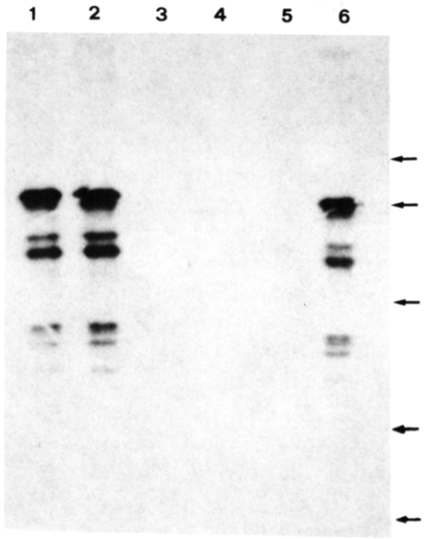


FIGURE 3: Direct interaction of ER with Zn(II) columns. Cytosol (70 mL) in buffer A was covalently labeled with [$^3$H]tamoxifen aziridine and subjected to immunoaffinity chromatography. The eluted material was dialyzed against buffer A containing 0.5 M KCl for 2 h at 4 °C, divided into aliquots, and charged onto iminodiacetate–Sepharose columns (2.5 mL) charged either with Zn(II), with Fe(II), or with nothing, respectively. The columns were washed with 10 mL of buffer A containing 0.5 M KCl and then eluted with 2.5 mL of EDTA, 50 mM. SDS–PAGE of the materials was carried out in 10% polyacrylamide slab gels that were then dried and fluorographed. Lane 1, BT from iminodiacetate–Sepharose (no metal charged); lane 2, BT from Fe(II)–iminodiacetate–Sepharose; lane 3, BT from Zn(II)–iminodiacetate–Sepharose; lane 4, eluate from iminodiacetate–Sepharose (no metal charged); lane 5, eluate from Fe(II)–iminodiacetate–Sepharose; lane 6, eluate from Zn(II)–iminodiacetate–Sepharose. Standards (indicated by arrows) were phosphorylase B (94.0 kDa), BSA (66.2 kDa), ovalbumin (42.7 kDa), and carbonic anhydrase (31.0 kDa).

were apparently irreversible because various treatments aimed at solubilizing them were unsuccessful. Radioactivity present in the shoulder of the elution profile sedimented as 8 S (not shown).

Imidazole gradient elution indicates that aggregation occurred under the above-mentioned chromatographic conditions. This is a handicap in the use of MCAC as a tool for ER purification, at least under our experimental conditions. Furthermore, it is possible that the adsorption of the receptor to these columns occurred through intermediate aggregating molecules. To confirm a direct interaction of the ER molecule with the metals, ER covalently labeled by [$^3$H]tamoxifen aziridine was purified by immunoaffinity chromatography. The immunoadsorbent was washed with buffer containing 1 M guanidine to remove nonreceptor molecules, regardless of association with ER. A predominant 67-kDa tamoxifen binding doublet molecule and other receptor fragments, also affinity labeled by tamoxifen, were eluted with ammonium hydroxide at pH 11.6. These fragments were constantly present even in the presence of protease inhibitors in the homogenization buffer. Under nondenaturing conditions, they were held together in an 8S conformation, as shown by analysis of the crude preparation on gel exclusion chromatography on Superose-6B. The eluate was dialyzed to permit refolding of the proteins denatured by the alkaline pH and then applied onto various metal-charged columns. The immunopurified ER bound to the same columns as ER from cytosol. This indicates a direct binding to the ER to the immobilized metal ions. Figure 3 shows the fluorography of an experiment of MCAC with immunopurified receptor. Purified ER passed through an uncharged or Fe(II)-charged column, while it was retained and recovered with EDTA from a Zn(II)-charged column.

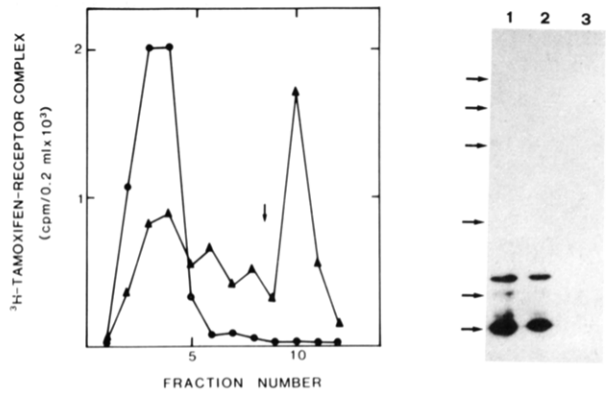


FIGURE 4: Interaction of ER fragments with Zn(II) columns. ER partially purified by DEAE-cellulose chromatography was covalently labeled with [$^3$H]tamoxifen aziridine. The sample was treated with 150 μg/mL trypsin at 20 °C for 15 min and then applied to a 180-mL Superose-6B gel filtration column in 0.5 M NaSCN. The peak of radioactivity (about 30 kDa) was dialyzed against 0.5 M KCl and chromatographed on the same column equilibrated with 0.5 M KCl in buffer A. The peak of radioactivity, eluted by the same volume as in the previous one, was applied onto iminodiacetate–Sepharose columns (3.5 mL) either charged with Zn(II) (▲) or not charged (●). Columns were washed with buffer A containing 0.5 M KCl prior to elution with 50 mM EDTA. The arrow indicates the application of EDTA (left panel). The pool of the peak of the Superose-6B column in 0.5 M KCl and the eluates from the iminodiacetate–Sepharose columns were analyzed by SDS–PAGE (12%) and then fluorographed. Lane 1, pool of the peak from Superose-6B in 0.5 M KCl; lane 2, eluate from Zn(II) column; lane 3, eluate from control column. Standards (indicated by arrows) were phosphorylase B (94.0 kDa), BSA (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31.0 kDa), and soybean trypsin inhibitor (21.5 kDa) (right panel).

In order to determine the molecular domain(s) in the ER engaged in this interaction, partially purified ER was labeled with [$^3$H]tamoxifen aziridine and extensively digested with trypsin. A solution of 0.5 M NaSCN was added to the trypsinized ER to fully dissociate receptor fragments held together by noncovalent interactions. The sample was then subjected to gel filtration chromatography through a Superose-6B column equilibrated in 0.5 M NaSCN. The affinity-labeled ER was eluted at a volume corresponding to a molecular weight of about 30 K. SDS–PAGE analysis of this peak revealed a group of fragments with a molecular weight of about 25K and other fragments of 14K or smaller (not shown). The eluted tamoxifen–ER complex was dialyzed to remove $SCN^-$ and was brought to 0.5 M KCl. To determine if removal of $SCN^-$ induced reassociation of labeled fragments with themselves or with other polypeptides, the dialyzed sample was again subjected to gel filtration through Superose-6B in the presence of 0.5 M KCl. The tamoxifen–ER complex eluted at the same volume as previously, indicating that no reassociation occurred. Analysis of the tamoxifen–ER complex under denaturing conditions gave similar results (Figure 4, right panel, lane 1). Finally, the sample was applied onto a Zn(II) column or onto an uncharged column. The tamoxifen–ER complex was significantly retarded by the column charged with Zn(II) as compared to the control column uncomplexed with metal ions. The inclusion of EDTA into the buffer determined the elution of a peak of tamoxifen–ER complex (Figure 4, left panel). SDS–PAGE analysis showed that all the fragments obtained by trypsinization were able to bind to the Zn(II) column (Figure 4, right panel, lane 2). This result suggests that the hormone binding domain of ER contains metal binding sequences.

*Inhibition of Estradiol Binding to the ER.* The above results indicated that the metal binding sites involved in the interaction with the immobilized metal ions were located in

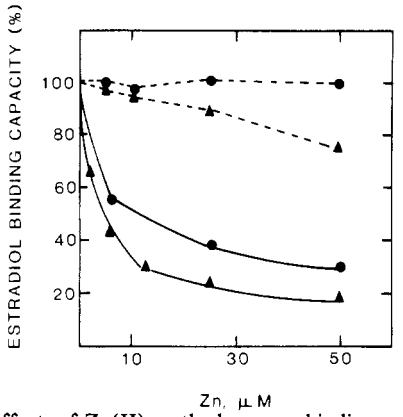


FIGURE 5: Effects of Zn(II) on the hormone binding capacity of the ER. Cytosol in buffer A, preincubated or not with [$^3$H]estradiol and treated with charcoal, was incubated for 1 h at 0 or 23 °C with $ZnCl_2$ at the indicated concentrations. At the end of incubation, [$^3$H]estradiol was added to the samples not preincubated and then treated with charcoal. Macromolecular bound radioactivity of all samples was measured. Prelabeled and incubated with $ZnCl_2$ at 0 °C (●---●) or at 23 °C (▲---▲); incubated with $ZnCl_2$ at 0 °C (●—●) or at 23 °C (▲—▲) and then labeled.

the hormone binding domain of the ER molecule. To test the effect of these metals on the hormone binding of ER in solution, the metals were added to the cytosol prior to incubation with the hormone. The same metal ions that interacted with the ER when immobilized on iminodiacetate–Sepharose strongly inactivated the hormone binding. Zn(II) and Ni(II) were the most effective; at 10 μM, they inhibited 50% of the hormone binding, whereas Co(II) and Cu(II) only had a comparable effect at 250 μM. As expected, Fe(II) and Cd(II) had no effect. Figure 5 shows that the inhibition occurred both at 0 °C and at 23 °C. This inhibitory effect was very slight if the metals were added after the preincubation with the hormone. A reverse plot of experiments performed at increasing concentrations of the hormone in the presence of 50 μM Zn(II) showed that the inhibition is noncompetitive, indicating that the metal binding site is not identical with the hormone binding site (not shown). The inhibition was not time dependent and was reversed by addition of EDTA to the cytosol after incubation with the metals and before incubation with the hormone (not shown). Only the inhibition by Cu(II) was partially irreversible. The irreversible inactivation induced by Cu(II), together with the results of MCAC with Cu(II) columns, is indicative of a different modification on the ER molecule induced by copper, compared to the other metals. Furthermore, it is a good test of comparison between the complexation chemistry in solid phase and in solution. In fact, the different behavior of the protein–metal interaction during the MCAC was also evident in the experiments in solution. The inhibition of the hormone binding was increased at increased ionic strength. The effect of the metal ions on the hormone binding of ER persisted after partial purification by various methods (preparative sucrose gradients, ammonium sulfate precipitation, and affinity chromatography on Sepharose–heparin). Furthermore, Zn(II) inhibited the hormone binding in the cytosol preparation extensively digested with trypsin (not shown). This is further evidence of the existence of metal binding sites in the steroid binding domain.

*Effects of Metals on the Nuclear Binding of the Hormone–ER Complex.* The nuclear retention in vitro of the steroid receptors reflects an alteration in the conformational state of the receptor, namely, its "transformation". It is promoted by the binding of the hormone (Puca et al., 1984; Denis et al., 1988) and corresponds in vivo to the binding of the complex itself to specific regulatory DNA sequences, followed by the transcription of the triggered genes.

Transformation in vitro can be achieved by high ionic strength, brief warming, dilution, and gel filtration (Milgrom et al., 1973; Cake et al., 1976). Over the past few years, various methods were developed to determine the shift from the untransformed to the transformed state of the receptor: DEAE ion-exchange chromatography, determination of the molecular weight, and assays of DNA, polyanions, or nuclear binding (Atger & Milgrom, 1976; Sakaue & Thompson, 1977; Holbrook et al., 1983). The correlation between the in vitro properties of the transformed receptor and the observations effectuated in vivo (Denis et al., 1988) supports the significance of these in vitro experiments.

Since receptor transformation appears to be related to the binding of the hormone (Denis et al., 1988) and since the metal binding sites described here are located at the hormone binding domain and interfere with the binding of the hormone, experiments were performed in order to determine the effect of the metals on the nuclear binding of the hormone–receptor complex. In these experiments, cytosol was preincubated with the hormone at 0 °C to avoid the inactivation of the hormone binding site by the metal.

At 5, 10, 25, and 50 μM, Zn(II) and Ni(II), but not Fe(II) and Cd(II) up to 250 μM, were able to inhibit the nuclear binding of cytosolic hormone–receptor complex. These experiments were carried out by bringing the prelabeled cytosol at 23 °C in buffer without molybdate, i.e., under transforming conditions, and contemporarily in the presence of isolated nuclei and of the metals. The inhibition obtained was the same as that in the hormone binding studies, suggesting that the effect both on the nuclear binding and on the hormone binding reflect the identical interaction of the metal with the receptor (not shown). This observation cannot determine which event was blocked by the metal. The metal might act during the transformation process, freezing the ER molecule at a pretransformed state, but it might also act after the transformation, by sterically modifying the transformed ER and keeping it from binding to the nucleus. The effect of the metals was therefore studied both on the transformation and on the nuclear binding of the transformed hormone–receptor complex. Figure 6 shows a DEAE-cellulose chromatography analysis of cytosol incubated under transforming conditions in the presence or absence of 50 μM $ZnCl_2$. When cytosol was preincubated with the hormone at 4 °C, i.e., in conditions in which transformation does not occur, ER eluted at an ionic strength of 250 mM KCl (Figure 6A), reflecting the native state of the receptor oligomeric complex. If the preincubation was performed at 23 °C, i.e., under transforming conditions, the peak of elution of ER shifts toward a major basicity (Figure 6B); 50 μM Zn(II) was very effective in blocking the p*I* shift observed during transformation (Figure 6C). Similar results were obtained in the presence of Ni(II) (not shown). Since another way to obtain the receptor transformation is by increasing the ionic strength in the absence of molybdate at 4 °C (0.4 M KCl in buffer B), the Zn(II) effect was tested on the KCl-induced transformation. Zn(II) was also effective in blocking the KCl-induced transformation (not shown). It was therefore evident that the transformation of the native receptor complex did not occur in the presence of Zn(II). These results suggest that the inhibition of the nuclear binding described above was surely determined by the block of transformation. Other experiments were carried out in which the cytosolic receptor was first transformed and then micromolar concentrations of Zn(II) were added. The transfor-

mation was obtained by warming the receptor for 1 h at 23 °C in the presence of 5 nM [$^3$H]estradiol. The transformed hormone–receptor complex was then brought to 4 °C and then, after the addition of micromolar concentrations of $ZnCl_2$, was incubated with isolated calf uterus nuclei for 60 min at 0 °C. The specific nuclear binding was performed as described under Experimental Procedures. Interestingly, Zn(II) was effective in blocking also the nuclear binding of the pretransformed hormone–receptor complex. If compared to a transformed hormone–receptor complex treated in the same way, except that Zn(II) was not added before incubating with the isolated nuclei, Zn(II) is able to reduce the nuclear binding to about 50% at 25 μM (Figure 7). These results indicate that the metal binding sites interfere with the nuclear interaction of the hormone–receptor complex by acting at both levels, either during the transformation or in impairing the binding of the pretransformed hormone–ER complex to nuclear structures.

## Discussion

The results strongly suggest a specific metal–protein interaction. The MCAC experiments performed with immunopurified ER clearly showed that this interaction occurs directly between the estradiol binding subunit and the metals and that it is specific for some divalent metal ions: Zn(II), Ni(II), and Co(II) are able to form coordinative bindings with the metal binding sites on the ER, whereas other divalent metal ions are not. There is appreciable evidence that the metal binding site(s) described here is(are) distinct from the DNA fingers and that they are located in the steroid binding domain of the ER molecule.

The primary sequence of steroid receptors appears to consist in various domains: N-terminal, DNA binding and hormone binding domains. These regions are highly compact and resistant to proteolytic degradation, whereas the hinge regions separating them are rich in basic amino acids and more likely to be exposed to proteolytic attack. This is particularly true for the one between the hormone binding and the DNA domain. In effect, proteolytic cleavage of various steroid receptors at the steroid binding site releases a fragment, called the meroreceptor, of 20–28 kDa. This is the smallest form of steroid receptor still containing an intact hormone binding site (Katzenellenbogen et al., 1987). Experiments with deletion mutants (Kumar et al., 1986) show that the C-terminal domain alone is sufficient to retain the hormone binding activity.

The results of the MCAC experiments performed with trypsinized ER, previously labeled with [$^3$H]tamoxifen aziridine, indicate that the fragments still retaining [$^3$H]tamoxifen aziridine, even the smaller ones (maximum molecular weight 15K), are able to bind to Zn(II) columns. Since [$^3$H]tamoxifen aziridine interacts with the hormone binding domain, it is conceivable that the fragments obtained by proteolytic cleavage of ER correspond to the meroreceptor or even smaller polypeptides. Furthermore, the C-terminal domain of ER contains various potential metal binding sites, especially residues 373–381 (HDQVHLLEC) and 513–530 (HIRHMSNKGMEHLYSMKC) for the human ER. The primary sequence presents histidine residues, and the elution of ER by imidazole from Zn(II) columns indicates the involvement of histidine residues in metal binding.

The presence of metal binding sites in the hormone binding domain of ER implies their possible influence on functional properties of the ER. In effect, Zn(II), Ni(II), and Co(II) inactivate the hormone binding site in a noncompetitive manner, indicating that the residues involved in metal binding are not the same as those involved in binding, though located at the same domain. Moreover, the same metals inhibit the

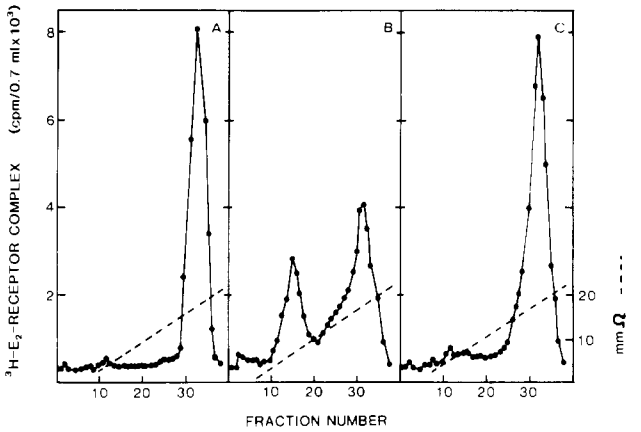


FIGURE 6: Effect of Zn(II) on the ER transformation. Aliquots of cytosol (2 mL) were incubated with [$^3$H]estradiol for 90 min at 0 °C (panel A) or at 23 °C in the absence (panel B) or presence (panel C) of 50 μM $ZnCl_2$. EDTA (final concentration 2 mM) was added to the samples, incubated for 1 h at 0 °C, and dialyzed in buffer A by Sephadex G-25 chromatography. Finally, samples were applied to DEAE-cellulose columns (6 mL) equilibrated in buffer A. Columns were washed with 10 bed volumes of buffer A and eluted with a 0–0.4 M KCl gradient. Fractions of 2 mL were collected.

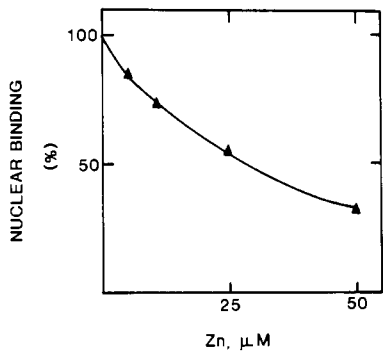


FIGURE 7: Effect of Zn(II) on the nuclear binding of the transformed hormone–receptor complex. Aliquots of cytosol (about 100000 cpm) in buffer B were transformed by [$^3$H]estradiol for 90 min at 23 °C and then incubated at 0 °C with $ZnCl_2$ at the indicated concentrations for 30 min. Finally, the nuclear binding was determined as indicated under Experimental Procedures.

ER transformation triggered by the hormone. This is a further indication that the metal binding sites are located in the steroid binding domain.

The fact that some metals can block ER transformation and inhibit the nuclear binding of a pretransformed hormone–receptor complex suggests a novel mechanism of regulation downstream to the binding of the hormone. In contrast with the DNA fingers, where metal binding seems to be essential to maintain receptor functionality (Sabbah et al., 1987), these other sites are negative modulators of the receptor activity.

Other authors found a slightly different effect of metals on the nuclear binding of the androgen receptor (Colvard & Wilson, 1984). Even though these metals have the same inhibitory effect on the hormone binding of both the androgen receptor and ER, the nuclear binding of the former was enhanced, rather than blocked, in the presence of Zn(II) and Ni(II). These differences could reflect different behaviors of the various steroid receptors or could be ascribed to the different experimental conditions used. In the above-mentioned work, a partially purified 4S form of the androgen receptor was used for the nuclear binding assay.

The existence of nuclear localization signals in the hormone binding domain of the glucocorticoid receptor was recently

reported (Picard & Yamamoto, 1987). A novel regulatory mechanism of steroid receptor action, in which metals can play an inhibitory role, can therefore be speculated. In this system, the hormone binding domain, though not directly involved in the binding to the steroid response elements, could interact with other components of the transcription machinery, influencing their action (Kumar et al., 1987).

Even though it seems unlikely that in physiological conditions one of these metals could be firmly associated with the ER molecule in sites other than the DNA binding domain (in this case, treatment of cytosol with EDTA would increase the number of hormone binding sites), it is interesting to consider the possibility of the existence of metal-dependent regulatory sites. It is worthwhile stressing that the micromolar concentrations of Zn(II) responsible for those effects on ER activity are consistent with a physiological role of this interaction.

Metal ions can stabilize the protein structure in an active or inactive conformation. The results presented suggest a receptor "mummification" induced by the metals. Even though Zn(II) is generally an activating cofactor of several enzymes, a few unusual examples of negative control by Zn(II) have been described. Zn(II) interacts with the nerve growth factor serine protease and inhibits the autocatalytic activation of the zymogen (Young & Koroly, 1980). Phosphotyrosine phosphatase is inhibited by micromolar concentrations of Zn(II) (Brautigan et al., 1981). The ER could be an unusual enzyme itself. Our group reported that in vitro "transformation" of ER was inhibited by serine proteases inhibitors (Puca et al., 1986). Some evidence supporting the hypothesis of enzymatic properties of the ER will be presented elsewhere (unpublished results).

In order to maintain homeostasis and prevent overgrowth, the division of normal cells must be tightly controlled. It is possible that unexplored feedback loops limit the proliferative stimulus and that the bound hormone in some conditions can fail to activate a cellular response. For the correct completion of this regulatory role, switches which are distinct from the hormone are conceivable, and the metal binding sites can be one of these molecular switches of the receptor action.

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**Registry No.** Zn, 7440-66-6; Ni, 7440-02-0; Co, 7440-48-4; Cu, 7440-50-8; estradiol, 50-28-2; progesterone, 57-83-0.

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# Tryptophan Fluorescence as a Probe of Placental Ribonuclease Inhibitor Binding to Angiogenin†

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ABSTRACT: The binding of human placental ribonuclease inhibitor (PRI) to angiogenin, a human protein that induces neovascularization, occurs with a 1:1 stoichiometry and is accompanied by a 50% increase in tryptophan fluorescence. In contrast, the binding of PRI to bovine pancreatic RNase A or to angiogenin oxidized at its single tryptophan residue results in a quenching of fluorescence. These observations suggest that there is a change in the local environment of Trp-89 of angiogenin. Quenching experiments with acrylamide are consistent with the view that Trp-89 is exposed in the native protein and becomes less accessible upon formation of the complex with PRI. Stopped-flow kinetic measurements monitoring the fluorescence enhancement indicate a two-step mechanism for the binding of PRI to angiogenin. The first step involves rapid formation of an enzyme·inhibitor complex, EI, followed by a slower isomerization of EI to a tight enzyme·inhibitor complex, EI*:

$$E + I \overset{K_1}{\rightleftharpoons} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI^*$$

In 0.1 M NaCl at pH 6 and 25 °C, the values of $K_1$ and $k_2$ are 0.53 $\mu$M and 97 $s^{-1}$, respectively. The apparent second-order rate constant of association at protein concentrations $\ll K_1$ is approximated by $k_2/K_1$ and equals $1.8 \times 10^8$ $M^{-1}$ $s^{-1}$. The corresponding value for the association of PRI with RNase A is only slightly higher, $3.4 \times 10^8$ $M^{-1}$ $s^{-1}$. The effects of pH and sodium chloride concentration on the association rate of PRI with angiogenin suggest the importance of ionizable groups and ionic interactions, respectively, in the association process. Increasing the pH from 5.5 to 9 decreases the apparent second-order rate constant of association of PRI with angiogenin 13-fold; increasing the sodium chloride concentration from 75 mM to 1 M decreases it 140-fold.

Several angiogenic substances have been isolated recently (Vallee et al., 1985; Folkman & Klagsbrun, 1987). Little is currently known, however, about the regulation of these substances. Angiogenin is a potent blood vessel inducing protein that has been obtained from both the conditioned medium of the human adenocarcinoma cell line HT-29 and normal human plasma (Fett et al., 1985; Shapiro et al., 1987). It is remarkably homologous to RNase A[1] and, indeed, exhibits ribonucleolytic activity (Strydom et al., 1985; Shapiro et al., 1986a; St. Clair et al., 1987). An RNase inhibitor isolated from human placenta (PRI) (Blackburn et al., 1977) inhibits both the angiogenic and ribonucleolytic activities ($K_i$ < 0.1 nM) of angiogenin (Shapiro & Vallee, 1987). Thus, PRI may play a critical role in their physiological control.

PRI is a member of a family of RNase inhibitors that occur in the tissues of many mammalian species (Roth, 1967; Blackburn & Moore, 1982). Other members of this family have been purified to homogeneity from bovine brain and liver and porcine, ovine, and rodent livers (Burton et al., 1980;

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; PRI, placental ribonuclease inhibitor; HSA, human serum albumin; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; C>p, cytidine cyclic 2′,3′-phosphate; CpG, cytidylyl(3′–5′)guanosine; UpG, uridylyl(3′–5′)guanosine; CD, circular dichroism, HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; DMS, dimethyl sulfide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; *n*, number of determinations.

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