The Binding Activity of Estrogen Receptor to DNA and Heat Shock Protein (Mr 90,000) Is Dependent on Receptor-bound Metal*

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1,10-Phenanthroline inhibited the DNA-cellulose binding of the transformed calf uterus estrogen receptor (homodimer of 66-kDa molecules: 5 S estrogen receptor) in a temperature- and concentration-dependent manner. This result appears related to the metal-chelating property of 1,10-phenanthroline, since the inhibition was decreased by addition of Zn²⁺ and Cd²⁺, but not by Ca²⁺, Ba²⁺, or Mg²⁺ for which the affinity of the chelator is low. Only a slight inhibition was observed in the presence of the 1,7-phenanthroline, a nonchelating analogue. After dialysis or filtration to remove free 1,10-phenanthroline, DNA binding of the 5 S estrogen receptor was still inhibited. Conversely, the chelator was unable to release prebound 5 S estrogen receptor from DNA-cellulose. The 5 S estrogen receptor DNA binding was inhibited when 1,10-phenanthroline was present during the transformation to activated receptor of the hetero-oligomeric nontransformed 9 S estrogen receptor, in which the hormone binding subunits are associated with heat shock protein, Mr 90,000 (hsp 90) molecules. In contrast, if 1,10-phenanthroline was removed before the transformation took place, only a slight inhibition was observed. Other experiments with EDTA indicated a similar inhibition of DNA-cellulose binding by the 5 S estradiol receptor, and all metal ions chelated by this agent prevented its inhibitory effect. The results indicate that 1,10-phenanthroline inhibited the DNA binding of the transformed 5 S estradiol receptor by chelating metal ion tightly bound to the receptor, which is not accessible to the chelator when the receptor is bound to DNA or to hsp 90. Therefore, they suggest that the metal ion may play a critical role in the interaction with DNA and hsp 90 by maintaining the structural integrity of the implicated receptor domain.

The molecular mechanisms by which steroid hormone-receptor complexes control the regulation of specific genes are poorly understood. It has been proposed that, following the binding of hormone, the receptor binds to specific DNA promoter elements leading to an activation of the transcription (1). Recently human and chick estradiol receptor and human glucocorticoid receptor cDNAs have been cloned and fully sequenced (2-7), and the corresponding primary amino acid structures were determined. A remarkably conserved cysteine-histidine-rich sequence has been proposed as the DNA binding region (5, 8). It is strikingly homologous to that determined for the partially sequenced chick progester-

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One receptor cDNA (9, 10) and is also present in v-erb A gene product (5). Cysteine-histidine-rich sequences have recently been reported to participate in the nucleic acid binding regions of several other proteins (11, 12) and shown to be Zn²⁺ binding domains. Since the putative DNA binding sequence of estrogen receptor could potentially be a metal binding domain (9), we have investigated the effect on estrogen receptor of chelating agents. Several studies have already suggested that the steroid hormone receptors may be metalloproteins (13-16).

We report here the effects of 1,10-phenanthroline on the molecular structure and on the binding of estradiol receptor to DNA-cellulose. Our data suggested that a metal (probably Zn²⁺) is involved directly in the DNA binding domain of the estrogen receptor; this also leads to further understanding of the relationship between the nontransformed hetero-oligomeric 9 S estrogen receptor in which two hormone binding units are associated with the heat shock protein (hsp 90) (17) and the transformed 5 S estradiol receptor form, which is a homodimer of the hormone binding subunit (18).

EXPERIMENTAL PROCEDURES

Materials—The following compounds were used: [6,7-³H]estradiol (specific activity, ~78 Ci/nmol) was from CEA (Gif sur Yvette, France); estradiol was a gift from Roussel-Uclaf (Romainville, France). Native DNA-cellulose and cellulose were obtained from Pharmacia (Uppsala, Sweden). 1,10-Phenanthroline was from Sigma and 1,7-phenanthroline from Alpha (Danvers, MA). ZnCl₂ was from Sigma and the other reagents were purchased from Merck (Darmstadt, Federal Republic of Germany).

Preparation of Cytosol—Calf uteri were homogenized in ~3 volumes of TG buffer (50 mM Tris-HCl, 12 mM α-thioglycerol, 10% glycerol, pH 7.5 at 25 °C). The cytosol was obtained as previously described (19).

Receptor Binding Activity Measurement—Samples containing receptor were incubated for 2-4 h with 10 nM [3H]estradiol at 0-4 °C. Excess unbound steroid was removed by incubation with a charcoal (0.5%) dextran T 80 (0.05%) suspension in TG buffer for 4 h at 4 °C. After centrifugation, the supernatant was counted, the background of nonspecific binding being determined by isotopic dilution. In the cytosol, receptor binding activity and protein concentration were 3-5 pmol/ml and 8-10 mg/ml, respectively.

DNA-cellulose Binding Assay—Samples containing [3H]E₂-5 S ER, treated as indicated in the text, were diluted to 0.1 mM KCl, added to 100 μl of packed DNA-cellulose or cellulose, and incubated for 1 h at 0 °C. After centrifugation, the supernatants were discarded and the pellets washed with 3 × 1 ml of TG buffer. The pellets were resuspended in 10 ml of scintillation mixture (Ready-Solv, Beckman Instruments) and counted. Specific binding to DNA-cellulose was calculated after subtraction of the radioactivity bound to unsubstituted cellulose (20).

Density Gradient Ultracentrifugation—Glycerol gradients were prepared and ultracentrifugation performed as described in Redeuilh et al. (21), except as noted in the text.

The abbreviations used are: hsp 90, heat shock protein of Mr = 90,000; ER, estrogen receptor.
RESULTS

Inhibition of the DNA Binding Activity of the Transformed Estrogen Receptor by 1,10-Phenanthroline—The effect of 1,10-phenanthroline on the binding of the transformed [3H]E2-5 S ER to DNA-cellulose was temperature-dependent (Table I). When the transformed [3H]E2-5 S ER was incubated for 30 min with 3 mM 1,10-phenanthroline at 0 °C, the DNA-cellulose binding was inhibited by 71%, while at 25 °C, 95% inhibition was observed. The inhibitory effect of 1,10-phenanthroline on the DNA binding of the receptor also was concentration-dependent (Fig. 1). It is unlikely that this inhibition can be attributed to a nonspecific hydrophobic interaction with the receptor, since the analogue 1,7-phenanthroline is not an effective inhibitor (Fig. 1). EDTA, a structurally different metal-binding agent, also markedly inhibited the DNA binding of [3H]E2-5 S ER. Half-inhibition was achieved at 25 °C with a concentration of ~0.4 mM 1,10-phenanthroline or 0.7 mM EDTA. At the concentrations used, both metal-chelating agents had no effect on the hormone binding activity of the receptor (data not shown). DNA binding was also decreased by dialysis of [3H]E2-5 S ER against buffer containing 1,10-phenanthroline or EDTA (Fig. 2). After removal of free 1,10-phenanthroline either by dialysis or by gel filtration, the DNA binding activity was not restored (data not shown). Another experiment showed that 1,10-phenanthroline did not affect the binding when it was added at the same time as the transformed [3H]E2-5 S ER complexes to the DNA-cellulose. It was also unable to release prebound [3H]E2-5 S ER from the DNA-cellulose (Fig. 2). The effect of 1,10-phenanthroline or EDTA on the binding of the transformed [3H]E2-5 S ER to DNA-cellulose was partially relieved in the presence of various divalent cations (Fig. 3). When Zn²⁺ or Cd²⁺, known

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**TABLE I**

Temperature dependence of the DNA binding inhibition of the transformed 5 S ER by 1,10-phenanthroline

| Temperature (°C) | Binding to DNA-cellulose |
|-----------------|--------------------------|
|                 | Control | 1,10-Phenanthroline |
| 0               | 174     | 50 (29%)          |
| 25              | 179     | 9 (5%)            |

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**FIG. 1.** Effect of 1,10-phenanthroline, 1,7-phenanthroline, or EDTA on the binding of [3H]E2-5 S ER to DNA-cellulose. Samples containing [3H]E2-5 S ER were incubated for 30 min at 25 °C with different concentrations of 1,10-phenanthroline (O), 1,7-phenanthroline (O), or EDTA (s). DNA-cellulose binding was performed as described under “Experimental Procedures,” and results are expressed in percent of total [3H]E2-5 S ER bound to DNA-cellulose. In the absence of 1,10-phenanthroline, approximately 54% (185 fmol) of the [3H]E2-5 S ER was bound to DNA-cellulose.

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**FIG. 2.** Effect of several treatments on the DNA binding activity of the [3H]E2-5 S ER. Cytosol containing [3H]E2-5 S ER was treated as indicated, and the DNA binding activity was measured as described under "Experimental Procedures." In these experiments, the [3H]E2-5 S ER was dialyzed against TG buffer or TG buffer containing 1 mM EDTA or 3 mM 1,10-phenanthroline (1,10-phen), respectively. In studies on the release of prebound S ER to DNA-cellulose, the S S ER DNA-cellulose was incubated for 30 min at 25 °C with 3 mM 1,10-phenanthroline, centrifuged after washing with 3 × 1 ml of TG buffer, and the DNA-cellulose pellet was counted as for the DNA-cellulose binding assay.

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**FIG. 3.** Influence of several divalent cations on the DNA binding inhibitory effect of 1,10-phenanthroline or EDTA. Samples containing [3H]E2-5 S ER were incubated for 30 min at 25 °C with 3 mM 1,10-phenanthroline (1,10-phen) or 1 mM EDTA in the absence or presence of 1 mM ZnCl₂, CdSO₄, BaCl₂, CaCl₂, or MgCl₂. DNA-cellulose binding was performed as described under "Experimental Procedures."
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to be chelated with high affinity by 1,10-phenanthroline, was added with the 1,10-phenanthroline partial inhibition of the subsequent decrease of DNA binding activity of \([^{3}H]E_{2-5}\) S ER was observed, while Ba\(^{2+}\), Ca\(^{2+}\), and Mg\(^{2+}\) were ineffective. In contrast, all these metal ions, which are chelated by EDTA, prevent the inhibitory effect of EDTA. These results clearly suggest that the transformed \([^{3}H]E_{2-5}\) S ER contains a metal ion (probably of the transition IIB group), which is required for the binding to DNA-cellulose and is removed by metal-chelating agents.

Effect of 1,10-Phenanthroline on the Nontransformed Estrogen Receptor—Molybdate-stabilized nontransformed estrogen receptor (\([^{3}H]E_{2-9}\) S ER) is converted to a molecular state able to bind DNA-cellulose when incubated for 45 min at 28°C in high salt medium (>0.4 M KCl) (Table II). Conversely when \([^{3}H]E_{2-9}\) S ER complexes were preincubated for 30 min at 25°C with 3 mM 1,10-phenanthroline and then transformed by KCl in the continued presence of the chelator, the \([^{3}H]E_{2}\) ER did not bind to DNA-cellulose. However, if the chelating agent was removed by dialysis before the transformation step, DNA binding was 66% of the control value. The residual inhibitory activity may be due to incomplete removal of 1,10-phenanthroline. If the resulting transformed \([^{3}H]E_{2-5}\) S ER was reincubated with 1,10-phenanthroline, its binding to DNA was completely inhibited.

We conclude that the estradiol receptor contains a metal ion refractory to chelating agent action when the receptor is in the nontransformed 9 S molecular form.

Molecular Size—The molybdate-stabilized nontransformed estradiol receptor complexes sediment as a sharp 9.3 S in low salt molybdate-containing glycerol gradient (Fig. 4A). Treatment with 1,10-phenanthroline at 25°C for 30 min did not affect this sedimentation pattern (Fig. 4A). The transformed estradiol receptor sediments as a 5.2 S peak in 0.4 M KCl containing glycerol gradients (Fig. 4B). After treatment with 1,10-phenanthroline, the receptor tended to aggregate. This aggregation was not observed after incubation, in the same conditions, with 1,7-phenanthroline (Fig. 4B).

As shown in Fig. 5, the transformed \([^{3}H]E_{2-5}\) S ER dialyzed against molybdate-containing low salt buffer in order to remove KCl could be partially (~40% of the original hormone binding activity) reconverted to a 9 S molecular form. In contrast, the 1,10-phenanthroline-treated estrogen receptor remained under aggregated form.

These results suggested that the estradiol receptor contains a metal ion which may be crucial in maintaining its conformational integrity.

**Table II**

Effect of 1,10-phenanthroline on the molybdate-stabilized ER

Cytosol containing \([^{3}H]E_{2-9}\) S ER stabilized by sodium molybdate was treated as indicated. The transformation of \([^{3}H]E_{2-9}\) S ER was performed by incubation at 28°C for 45 min in high salt medium (0.4 M KCl); two dialysis experiments were performed against TG buffer containing 20 mM sodium molybdate. The abbreviations used are: 1,10-phen, treatment with 3 mM 1,10-phenanthroline for 30 min at 25°C. The abbreviation transformation is defined as an increase in DNA binding activity observed 45 min after treatment with 3 mM 1,10-phenanthroline or 1,7-phenanthroline. The values in parentheses represent the percent of the native hormone binding. The values in parentheses represent the percent of the 9 S ER bound to DNA-cellulose compared with their respective controls in the absence of 1,10-phenanthroline.

| Treatment | Amount of \([^{3}H]E_{2}\) ER | Total Bound | % of total bound to DNA-cellulose |
|-----------|--------------------------|-------------|----------------------------------|
| 9 S ER    | Transformation 406 210 | 51.7        |                                  |
| 9 S ER + 1,10-phen | Transformation 400 20 | 5 (9.6)*  |
| 9 S ER    | 1) Dialysis 430 272 | 63.2        |                                  |
|           | 2) Transformation 365 179 | 49 (65.8)  |
| 9 S ER + 1,10-phen | 1) Dialysis 360 8 | 3 (2.9)     |
|           | 2) Transformation 1,10-phen |

* Values in parentheses represent the percent of the 9 S ER bound to DNA-cellulose compared with their respective controls in the absence of 1,10-phenanthroline.
the inhibition was observed if 1,10-phenanthroline is present during the transformation (9 S to 5 S) but not if it is removed before the transformation step. These observations suggest that the metal ion is bound by hormone binding units and becomes accessible to chelating agents after release of the hsp 90 which occurs during the transformation. When the receptor is associated with hsp 90, in the 9 S molecular form, the metal ion is located in a protein environment inaccessible to chelator.

Several experiments have been performed in order to analyze the mechanism underlying the inhibition of receptor DNA binding. Addition of metal ion, during the course of inhibition, had no effect, even when carried out under an N₂ atmosphere or in the presence of NaBH₄. The failure of metal ion to reduce the inhibitory effect of chelating agents argues against the expression of a receptor-chelator complex. It is, therefore, probable that metal ion is removed from the 5 S estradiol receptor by the chelator. Failure of reducing conditions to restore DNA binding argues against the involvement of free cysteine oxidation being responsible for the change. It is, therefore, supposed that an irreversible conformational change in the protein occurs as a result of metal ion removal. Ultracentrifugation studies support the hypothesis that metal ion plays a critical role in maintaining the structural integrity of the receptor, since (1) no significant change of the 9 S estradiol receptor could be detected as a result of the 1,10-phenanthroline treatment, whereas the transformed 5 S estradiol receptor tended to aggregate; (2) treatment of 5 S estradiol receptor by 1,7-phenanthroline did not influence the sedimentation pattern; (3) the transformed 5 S estradiol receptor could be partially reconverted to 9 S estradiol receptor, but not when treated by 1,10-phenanthroline after which aggregation was observed.

In conclusion, these studies provide evidence for the presence of metal ion in the estradiol receptor in association with the homodimer 5 S estradiol receptor. The M⁺⁺ can be removed from 5 S estradiol receptor by metal-chelating agents, and the receptor is then no longer able to bind to DNA. The fact that excess 1,10-phenanthroline is unable to release 5 S estradiol receptor prebound to DNA-cellulose suggests that the metal ion is located in the nucleic acid binding domain of the estradiol receptor as defined by amino acid sequence. In the nontransformed 9 S estradiol receptor structure, the metal ion is located in a protein environment inaccessible to chelating agent. We have found that the hetero-oligomer includes two molecules of both 65-kDa estradiol receptor and hsp 90 (17). Therefore, M⁺⁺ may serve a structural role in maintaining the conformational structure of binding subunits involved in the protein-protein interaction between the hormone binding subunits and hsp 90, which is a common feature of all nontransformed "S receptors" (23, 24). A potentially effective of Zn⁺⁺ on the stabilization of the 8 S androgen receptor had been previously reported (25).

Zinc is known to be an integral component of numerous DNA binding proteins (11, 12), and it seems likely that similar metal binding domains are discrete structural units found in this group of proteins.

### DISCUSSION

Metal chelators, mainly 1,10-phenanthroline, have been used to investigate the possible presence and functional role of a metal ion in the structure of estrogen receptor.

The results clearly demonstrate that treatment of the transformed 5 S estradiol receptor with 1,10-phenanthroline prevents the binding of [³H]E₂-5 S ER complex to DNA-cellulose in a concentration- and temperature-dependent manner. The effect of 1,10-phenanthroline may be related to its chelating properties, since it can be partially prevented by addition of metal ions such as Zn⁺⁺ and Cd⁺⁺, but not by Ca⁺⁺, Ba⁺⁺, or Mg⁺⁺ which have a low affinity for the chelating agent (22). Furthermore, the inhibitory effect is not observed using the nonchelating isomere 1,7-phenanthroline. The inhibition mediated by 1,10-phenanthroline remains after extensive dialysis or gel filtration showing that the inhibition is not dependent on the presence of free chelator in the medium. The inhibition of DNA binding can also be observed with EDTA, Zn⁺⁺, Cd⁺⁺, Ba⁺⁺, Ca⁺⁺, or Mg⁺⁺, which are all chelated by EDTA (22), prevent the inhibitory effect. Therefore, the data suggest that the inhibition of DNA binding activity of the 5 S estradiol receptor is due to the chelation of a metal ion, probably of the transition IIIB group. It is believed that the metal ion involved is part of the 9 S estradiol receptor structure, since the inhibition was observed if 1,10-phenanthroline is present during the transformation (9 S to 5 S) but not if it is removed before the transformation step. These observations suggest that the metal ion is bound by hormone binding units and becomes accessible to chelating agents after release of the hsp 90 which occurs during the transformation. When the receptor is associated with hsp 90, in the 9 S molecular form, the metal ion is located in a protein environment inaccessible to chelator.

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