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A Ligand-Induced Conformational Change in the Estrogen Receptor Is Localized in the Steroid Binding Domain†

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ABSTRACT: Upon binding estrogen, the estrogen receptor (ER) is proposed to undergo some form of conformational transition leading to increased transcription from estrogen-responsive genes. In vitro methods used to study the transition often do not separate heat-induced effects on the ER from estrogen-induced effects. The technique of affinity partitioning with PEG-palmitate was used to study the change in the hydrophobic surface properties of the ER upon binding ligand with and without in vitro heating. Upon binding estradiol (E_2), the full-length rat uterine cytosolic ER undergoes a dramatic decrease in surface hydrophobicity. The binding of the anti-estrogen 4-hydroxytamoxifen (4-OHT) results in a similar decrease in surface hydrophobicity. These effects are independent of any conformational changes induced by heating the ER to 30 °C for 45 min. The use of the human ER steroid binding domain overproduced in *Escherichia coli* (ER-C) and the trypsin-generated steroid binding domain from rat uterine cytosolic ER demonstrates that the decrease in surface hydrophobicity upon binding E_2 or 4-OHT is localized to the steroid binding domain. Gel filtration analysis indicates that the change in surface hydrophobicity upon binding ligand is an inherent property of the steroid binding domain and not due to a ligand-induced change in the oligomeric state of the receptor. The decrease in surface hydrophobicity of the steroid binding domain of the ER upon binding E_2 or 4-OHT represents an early and possibly a necessary event in estrogen action and may be important for "tight" binding of the ER in the nucleus.

The steroid receptor superfamily of DNA binding, nuclear transcription factors includes the estrogen receptor (ER)[1] (Evans, 1988). Early studies using controlled proteolysis as well as more recent cloning of the cDNA for the ER have revealed distinct functional domains (Green et al., 1986;

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[1] Abbreviations: ER, estrogen receptor; hsp, heat shock protein; ERE, estrogen response element; E_2, estradiol; 4-OHT, 4-hydroxytamoxifen; PEG, poly(ethylene glycol); ATPP, aqueous two-phase partitioning; PMSF, phenylmethanesulfonyl fluoride; HAP, hydroxylapatite; EDTA, ethylenediaminetetraacetic acid; BCIP, 5-bromo-4-chloro-3-indolyl 1-phosphate; NBT, nitro blue tetrazolium; U, unoccupied ER; EO, E_2-occupied ER; AO, 4-OHT-occupied ER; UH, unoccupied heated ER; EOH, E_2-occupied heated ER; AOH, 4-OHT-occupied heated ER; DES, diethylstilbestrol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; TAMZ, tamoxifen aziridine; V_0, void volume; SEM, standard error of the mean; kDa, kilodalton(s).

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Greene et al., 1986; Koike et al., 1987; Krust et al., 1986; Sherman, 1984; White et al., 1987). The steroid binding domain is responsible for high-affinity binding of estrogen and has been implicated in transcriptional activation, protein dimerization, and interaction with heat shock protein 90 (hsp90) (Carson-Jurica et al., 1990). The DNA binding domain mediates ER binding to specific DNA enhancer sequences called estrogen response elements (ERE), proposed to localize the ER to its site of action in an estrogen-responsive gene. The functional roles of the amino terminus and hinge regions are less well understood, although the amino terminus may be involved in the transcriptional activation of some genes (Berry et al., 1990; Lees et al., 1989; Tora et al., 1988, 1989).

Estrogen binding to the unoccupied ER is the signal that leads to increased transcription from an estrogen-responsive gene. Both the unoccupied and ligand-bound ERs are localized within the nucleus (King & Greene, 1984; McClellan et al., 1984; Welshons et al., 1984). However, the unoccupied ER is easily extracted into hypotonic buffer (a cytosol), whereas the ligand-bound ER is held tightly in the nucleus and requires high salt for extraction (Grody et al., 1982; Horwitz & McGuire, 1978; Miyabe & Harrison, 1983).

The anti-estrogen 4-hydroxytamoxifen (4-OHT) acts as a competitive inhibitor of estradiol (E_2) (Jordan & Murphy, 1990). The 4-OHT–ER complex is bound tightly to the nucleus, as is the E_2–ER complex (Clark & Peck, 1979; Katzenellenbogen et al., 1979; Rochefort & Borgna, 1981). Several groups have shown that the unoccupied, E_2-bound, and 4-OHT-bound ER complexes all have similar binding affinities for an ERE (Curtis & Korach, 1990, 1991; Klein-Hitpass et al., 1989; Murdoch et al., 1990). The distinctive properties of the 4-OHT–ER complex that prevents the ER from becoming biologically active but brings about tight nuclear binding are unknown (Nelson et al., 1988).

Our laboratory previously used affinity partitioning in the presence of PEG–palmitate to demonstrate a significant decrease in the surface hydrophobicity of the rat uterine cytosolic ER upon binding E_2 (Hansen & Gorski, 1986). Studies using aqueous two-phase partitioning (ATPP) also showed, kinetically, that the heat-induced transitions may be different from ligand-induced changes (Hansen & Gorski, 1989).

The structural properties of the unoccupied ER that prevent tight nuclear binding and transcription from occurring are unknown, as is the functional significance of any structural changes induced by estrogen binding. Since estrogen binds in the steroid binding domain of the ER, we chose to investigate changes in the structure of the isolated steroid binding domain induced by binding estrogen or anti-estrogen. This study demonstrates that (1) the estrogen-induced decrease in surface hydrophobicity is localized to the steroid binding domain of the ER, (2) the 4-OHT–ER steroid binding domain complex demonstrates a decrease in hydrophobicity similar to the E_2–ER complex, and (3) the decrease in surface hydrophobicity upon binding hormone or antihormone is independent of the effects of heating in vitro. We hypothesize from these observations that the decrease in surface hydrophobicity of the steroid binding domain of the ER allows for tight nuclear binding of the ligand–ER complex and this represents an early and necessary event in steroid hormone action. A subsequent anti-estrogen-inhibited step may reflect nonhydrophobic changes in the structure of the ER or a change at other sites on the receptor that could be essential for transcriptional activation.

Materials and Methods

Dextran (M_r 480000), methoxypoly(ethylene glycol) (PEG, M_r 5000), trypsin, aprotinin phenylmethanesulfonyl fluoride (PMSF), Blue Dextran (M_r 2000000), and Sephacryl S200 were purchased from Sigma. 17β-[2,4,6,7-^{3}H]Estradiol (90–110 Ci/mmol), (*Z*)-4-[*N-methyl*-^{3}H]hydroxytamoxifen (80–100 Ci/mmol), and [*ring*-^{3}H]tamoxifen aziridine (10–30 Ci/mmol) were from New England Nuclear and Amersham. Immature Sprague-Dawley female rats (19 days of age) were from Harlan Sprague Dawley (Madison, WI). Bio-Gel HT hydroxylapatite (HAP) was from Bio-Rad Laboratories. PEG–palmitate and unsubstituted PEG (M_r 8000) from the same lot were obtained from Aqueous Affinity AB (Arlov, Sweden). Ready Safe scintillation cocktail was from Beckman. BCIP (5-bromo-4-chloro-3-indolyl 1-phosphate) and NBT (nitro blue tetrazolium) were from Promega. ENHANCE was from New England Nuclear. All other chemicals were reagent grade. All procedures were performed at 4 °C unless otherwise indicated.

Preparation of Rat Uterine Cytosolic ER. Preparation of rat uterine cytosolic ER has been previously described (Hansen & Gorski, 1985; Murdoch et al., 1990). In brief, uteri from 19-day-old rats were homogenized in 10 mM Tris-HCl (pH 7.5, 25 °C), 1.5 mM EDTA, and 10 mM mercaptoethanol (TEM) buffer at 3 uteri/mL and centrifuged at 436000*g* for 10 min to obtain cytosol containing unoccupied ER (U). The unoccupied ER was incubated with 5–10 nM ^{3}H-E_2 (EO) or ^{3}H-4-OHT (AO) for 1.5 h at 4 °C. Heated forms of the ER were obtained by placing the unoccupied or ligand-occupied (45 min at 4 °C) ERs at 30 °C for 45 min. The resulting ER forms were the unoccupied heated (UH), E_2-occupied heated (EOH), and 4-OHT-occupied heated (AOH) ERs. Parallel incubation with a 200-fold molar excess of unlabeled diethylstilbestrol (DES) was used to determine nonspecific binding for all ER forms.

Trypsin Generation of the Steroid Binding Domain of the Cytosolic ER. Stock trypsin solution (0.5 mg/mL) was made 3–5 h before use by dissolving solid trypsin in TEM at 4 °C. Similarly, a mixed stock solution of aprotinin (0.5 mg/mL) and PMSF (1.5 mM) was prepared in TEM at 4 °C. For each ER form, 1 μL of trypsin stock was mixed with every 100 μL of sample for 1 h at 4 °C, with the final trypsin concentration 5 μg/mL. One microliter of the aprotinin/PMSF mixture was added to every 100 μL of sample to inhibit trypsin activity, with the final concentrations 2.5 μg/mL aprotinin and 15 μM PMSF. This ratio of trypsin to protease inhibitor mix was demonstrated to completely inhibit trypsin activity (data not shown).

Preparation of the Overproduced Human ER Steroid Binding Domain. The human ER steroid binding domain was overproduced in *Escherichia coli* (Ahrens et al., 1990, 1992). The restriction fragment *Nae*I (nucleotide 954) in the ER cDNA sequence to *Eco*RI (3′ end of the coding region) from the HEO plasmid (Green et al., 1986) was inserted in-frame into the pIC-20H vector. The resulting ER-C plasmid was used to transform *E. coli* JM 109 cells. Expression of the mRNA encoding the carboxy-terminal end of the ER protein containing the steroid binding domain (ER-C) was regulated by the inducible *lac* promoter. Transformed bacteria were grown overnight in LB broth in the presence of 50 μg/mL ampicillin, induced with 1 mM isopropyl thiogalactoside (IPTG) for 30 min at 37 °C, and for 4 h at 18 °C. The bacteria were collected by centrifugation, resuspended in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 25% sucrose, 2 μg/mL aprotinin, 1 μg/mL leupeptin, 0.1 mM PMSF, and 0.1 mM

sodium metabisulfite, and lysed with lysozyme (2.5 mg/mL) on ice for 30 min. The ER-C protein was isolated in the form of inclusion bodies as previously described (Nagai et al., 1985), except the DNA was sheared by repeated sonication instead of DNase digestion. The inclusion bodies were dissolved in 8 M urea, 50 mM sodium borate (pH 9.2), and 1 mM dithiothreitol (DTT) at 4 °C, sonicated just prior to use, and dialyzed for 2 h at 4 °C against a 2000–4000-fold excess volume of dialysate containing 50 mM K_2HPO_4 (pH 7.4) and 1 mM DTT. Following dialysis, the steroid binding domain preparation was centrifuged in a microfuge for 1 min at maximum speed to pellet any particulate matter. The supernatant contained the ER-C preparation used for the experiments described in this paper. Several other proteins were present in this preparation as detected by Coomassie staining of a sample analyzed by SDS–PAGE (Laemmli, 1970) (data not shown).

Aqueous Two-Phase Partitioning. Phase systems were prepared as previously described (Hansen & Gorski, 1985) with the following changes. Dextran and methoxy-PEG were initially stored as 20–30% (w/w) stock solutions in double-distilled H_2O (ddH_2O) at 4 °C. Equilibrated phase systems composed of 7.67% (w/w) each of dextran and methoxy-PEG with 0.167 M K_2HPO_4 (pH 7.4) in TEM were premixed. Aliquots (0.9 mL) of this polymer and salt mixture were added to 0.6 mL of buffer and cytosol or ER-C preparation at 4 °C. The partitioning behavior of the ER is independent of the protein concentration (Hansen & Gorski, 1985). For cytosolic ER, 0.1–0.2 mL of cytosol (2–3 mg of protein/mL) and for the ER-C preparations 0.075 mL (1–3 mg of protein/mL) were used in the final partitioning system. The final phase system consisting of 4.6% dextran, 4.6% methoxy-PEG, 0.1 M K_2HPO_4 (pH 7.4), and cytosol or ER-C in TEM was vortexed for 10 s and then centrifuged for 5 min (600*g* at 4 °C) to form the two phases. Aliquots of each phase were sampled using a positive-displacement pipet and incubated with tritiated ligand for 1.5 h at 4 °C for the unoccupied and unoccupied heated ER forms or assayed using HAP immediately for the ligand-occupied ER forms. The phase H_2O content was determined as previously described (Hansen & Gorski, 1985) and consisted of 90–95% H_2O for the upper phase and 80–85% for the lower phase. For affinity partitioning experiments, the final phase concentration of PEG–palmitate was 10–20 μM. For ^3H-E_2- or ^3H-4-OHT-occupied samples partitioned in the presence of PEG-palmitate, the phase systems were adjusted to 5 nM with the respective tritiated ligand in order to optimize recovery of the occupied ER complex. For the unoccupied ER forms in the PEG–palmitate system, a similar final volume of ethanol (<0.1%) was added to the phase system. Adding 5 nM ^3H-E_2, ^3H-4-OHT, or ethanol to the phase system without PEG–palmitate had no effect on the K_{obs} values (data not shown). The partition coefficients (K_{obs}) for the various ER forms and their recoveries were calculated as previously described (Hansen & Gorski, 1985). The K_{obs} value is experimentally determined by calculating the concentration of ER in the upper phase and dividing this by the concentration of ER in the lower phase.

Hydroxylapatite Assay of the Phases. The HAP assay was performed as previously described (Hansen & Gorski, 1985; Murdoch et al., 1990) except that the HAP pellets after washing with buffer were extracted with 0.75 mL of ethanol at room temperature. Aliquots of 0.5 mL were counted in 3.5 mL of Ready Safe scintillation cocktail at 36–40% efficiency.

Saturation Binding Analysis. Aliquots of the ER-C preparation were diluted 20–40-fold into TEM buffer containing 0–25 nM ^3H-E_2 ± a 200-fold molar excess of unlabeled DES and incubated at 4 °C for 1.5 h. Each sample had 0.5 mL of 70% HAP added for 30 min at 4 °C with several mixings. The HAP was pelleted, and a 150-μL aliquot of the supernatant was mixed with 4 mL of scintillation fluid and counted to directly determine the concentration of free ^3H-E_2. The HAP pellets were processed as above to determine the amount of specific ^3H-E_2 bound. The equilibrium dissociation constant (K_d) was estimated using a nonlinear reiterative fit by the LIGAND computer program (Munson & Rodbard, 1980).

Immunoblotting. All samples were diluted 1:1 with 2× sample buffer (15% glycerol, 62.5 mM Tris-HCl, pH 6.8, 0.7 M mercaptoethanol, 3% SDS, and 0.05% bromophenol blue), boiled for 5 min, and stored prior to separation by SDS–PAGE at −20 °C. The separated proteins were transferred to nitrocellulose, and the ER bands were identified using affinity-purified polyclonal rabbit antibody ER712 (1:2500) raised against a synthetic peptide with a sequence derived from the D (hinge) region of the rat ER (Furlow et al., 1990). The second antibody was a goat anti-rabbit IgG (1:5000) linked to alkaline phosphatase, and the substrate (BCIP/NBT) was added for color development. The molecular mass standards were from Bio-Rad and included rabbit muscle phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), hen egg white ovalbumin (43 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and hen egg white lysozyme (14 kDa).

Affinity Labeling the ER with ^3H-Tamoxifen Aziridine (^3H-TAMZ). ER samples were covalently labeled with the anti-estrogen ^3H-TAMZ (Katzenellenbogen et al., 1983), and the proteins were then separated by SDS–PAGE. The gel was fixed using 10% (v/v) glacial acetic acid, 50% methanol, and 40% ddH_2O for 1 h at 25 °C, incubated in the fluor ENHANCE for 1 h at 25 °C, and incubated with cold ddH_2O for 25–30 min. The gel was placed on 3MM Whatman filter paper, covered with plastic wrap, and dried for 2.5 h under low heat (60 °C). The dried gel was exposed to XAR-5 film (Kodak) at −70 °C for 7–14 days.

Sephacryl S200 Gel Filtration Chromatography. Gel filtration was performed on a 1.2 × 47.5 cm column of Sephacryl S200 equilibrated in 10% glycerol, 50 mM KCl, 10 mM Tris-HCl (pH 7.5 at 25 °C), 1.5 mM EDTA, and 1 mM DTT at 4 °C. Samples were applied in a volume of 260 μL containing Blue Dextran (M_r 2 000 000) to mark the void volume (V_0). The column was eluted at a flow rate of 0.15 mL/min, and 0.35-mL fractions were collected. The column was calibrated using bovine IgG (M_r 152K; Stokes radius 5.2 nm) in the absence of DTT, dansylated albumin (M_r 66K; Stokes radius 3.6 nm), ovalbumin (M_r 43K; Stokes radius 3.05 nm), carbonic anhydrase (M_r 31K; Stokes radius 2.4 nm), and cytochrome *c* (M_r 12K; Stokes radius 1.65 nm). Bovine IgG, ovalubumin, carbonic anhydrase, and cytochrome *c* were detected by measuring the absorbance at 280 nm, whereas dansylated albumin was detected by fluorescence under a UV lamp. Ten milliliters of the void volume was collected in a graduated tube and discarded, and the fraction collector was started. For the ER-C preparation prelabeled with ^3H-E_2 ± a 200-fold molar excess of DES, the ^3H-E_2 binding activity was determined by HAP assay for each fraction. For the unoccupied ER-C sample, 0.3 mL of 10 nM ^3H-E_2 was added to each odd-numbered fraction, and 0.3 mL of 10 nM ^3H-E_2 plus a 200-fold molar excess unlabeled DES was added to each even-numbered fraction to determine the nonspecific binding, and the samples were incubated at 4 °C for 1.5 h. The ^3H-E_2 binding activity was determined using the HAP assay. The

specific ^{3}H-E_2 binding activity was calculated by subtracting the binding activity in the even fractions from the binding activity in the odd fractions.

Results

Affinity Partitioning with PEG–Palmitate of the Full-Length Rat Uterine Cytosolic ER. ATPP carried out in a PEG/dextran system in the presence of a small amount of PEG–palmitate can be used to gain information about the hydrophobic surface properties of a protein. A small amount of PEG esterified to palmitic acid partitions with the unsubstituted PEG creating a more hydrophobic environment in the upper phase (Axelsson, 1978; Axelsson & Shanbhag, 1976; Hansen & Gorski, 1986; Johansson, 1984; Shanbhag & Johansson, 1974, 1979; Walter & Johansson, 1986). Protein partitioning in PEG–palmitate-substituted phase systems is dependent upon hydrophobic interaction between the palmitate residue and the hydrophobic surface properties of the protein. In an ATPP system without PEG–palmitate, the ER distributes between the two phases, and this distribution can be quantitated by the K_{obs} value. The behavior of the ER within ATPP systems can be described by the equation (Albertsson, 1986; Hansen & Gorski, 1985; Walter & Johansson, 1986):

$$\ln K_{obs} = \ln K_0 + (ZF/RT)\Delta\Psi$$

where Z is the net molecular charge and $\Delta\Psi$ is the salt-dependent interfacial potential difference. The other terms are Faraday's constant (F), the gas constant (R), and the temperature (T) in kelvin. The term $(ZF/RT)\Delta\Psi$ represents the electrostatic contribution to the K_{obs} value of the interaction between the protein molecule and phase system. This electrostatic term is assumed to be constant between the unsubstituted and PEG–palmitate-substituted systems because the only difference between the two phase systems is the presence of the noncharged PEG–palmitate. Therefore, any difference in the measured K_{obs} is due to changes in K_0. K_0 is the contribution to the partition coefficient caused by nonelectrostatic surface interactions of the ER with the phase environment. PEG–palmitate remains in the upper phase, creating a more hydrophobic environment. When ER is partitioned into this ATPP system with PEG–palmitate, the ER distributes between the two phases on the basis of its surface properties, including its hydrophobicity with a resulting K'_{obs} value. If a protein's $K_{obs} = K'_{obs}$, there are minimal hydrophobic properties on the surface of the protein. However, $K'_{obs} > K_{obs}$ suggests the surface properties of the protein contain a large hydrophobic component.

Previous data from our laboratory demonstrated that the phase components have little effect on the physicochemical properties of the ER (Hansen & Gorski, 1985). Saturation binding analysis of the ER in the presence of the polymers (PEG and dextran) demonstrates that the binding affinity of the ER for ^{3}H-E_2 is unaffected by PEG or dextran (data not shown). Therefore, the polymers themselves do not alter the conformation or function of the ER's steroid binding domain. In general, both PEG and dextran are relatively inert and unlikely to interact directly with most proteins.

In the ATPP system without PEG–palmitate, the unoccupied cytosolic ER and the 4-OHT-bound ER had similar K_{obs} values, whereas the E_2-bound ER had a smaller K_{obs} value (Figure 1A). However, it should be noted that a significant difference between the K_{obs} value of the E_2-occupied and the 4-OHT-occupied ERs is still observed. Heating these various forms of the ER resulted in dramatic decreases in the K_{obs} values for all three. We then partitioned each of these ER forms into a phase system containing 10–20 μM PEG–palmitate. We confirmed that the PEG–palmitate in this ATPP system was in the upper phase by partitioning bovine serum albumin into the unsubstituted and substituted systems. Albumin had a 3-fold higher preference for the upper phase when PEG–palmitate was present than when it was not present (data not shown). The unoccupied ER partitioned preferentially into the PEG–palmitate-rich upper phase, whereas the E_2-occupied ER showed no change in K_{obs} from the unsubstituted system. This suggests an E_2-induced conformational change in the ER, resulting in the "burying or masking" of hydrophobic residues present on the surface of the unoccupied ER. When the 4-OHT–ER complex was partitioned in the PEG–palmitate phase system, the K_{obs} value was significantly less than the K_{obs} value of the unoccupied receptor. However, a comparison of the K_{obs} values for the unsubstituted and the PEG–palmitate-substituted systems for the 4-OHT-occupied ER shows no significant change in the K_{obs} value. Thus, the anti-estrogen-occupied complex undergoes a loss in surface hydrophobic residues able to interact with PEG–palmitate, similar to the estrogen-occupied complex.

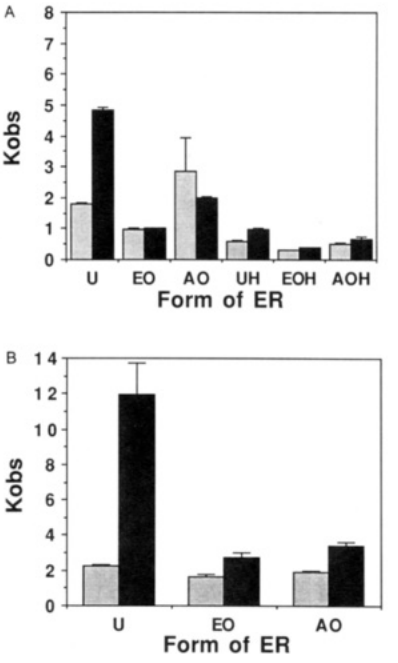

FIGURE 1: (A) Determination of the K_{obs} values for several forms of the intact rat uterine cytosolic ER partitioned in the absence (stippled bars) and in the presence (solid bars) of PEG–palmitate. The forms of the rat uterine cytosolic ER used are as follows: U, unoccupied ER kept at 4 °C; EO, E_2-occupied ER kept at 4 °C; AO, 4-OHT-occupied ER kept at 4 °C; UH, unoccupied ER heated to 30 °C for 45 min; EOH, E_2-occupied ER heated to 30 °C for 45 min; AOH, 4-OHT-occupied ER heated to 30 °C for 45 min. The values shown represent the means ± the standard error of the mean (SEM) for two separate determinations. The percent recoveries of the ER ranged from 35 to 90%. (B) Determination of the K_{obs} values for the three nonheated forms of the steroid binding domain of human ER overproduced in *E. coli* (ER-C) partitioned in the absence (stippled bars) and in the presence (solid bars) of PEG–palmitate. Following dialysis against 50 mM K_2PO_4 and 1 mM DTT, the overproduced steroid binding domain was treated as in (A), U, EO, and AO. Values represent the means ± SEM for six to eight separate determinations, and the percent recoveries ranged from 36 to 95%.

In order to establish that these changes in hydrophobicity represented true hormone-induced changes in the ER and not changes in other proteins bound to the ER, we partitioned the heated forms of the ER into PEG–palmitate. Heating the unoccupied, E_2-occupied, and 4-OHT-occupied ERs at 30 °C for 45 min results in the loss of hsp90 proteins and transformation to the DNA binding form of the ER (Carson-Jurica

et al., 1990). However, other proteins, including hsp70, probably remain bound to the ER after heating as has been demonstrated for the progesterone receptor (Smith et al., 1990). Figure 1A shows that heating these forms of the ER and partitioning into PEG–palmitate result in effects similar to the nonheated forms. The unoccupied–heated ER moves into the upper phase, although the magnitude of the effect is not as great as for the unoccupied, nonheated ER. The E_2- and 4-OHT-occupied–heated ERs showed little change in K_{obs} values in the PEG–palmitate phase system. Thus, heating the cytosol, which changes several physical properties of the ER (Grody et al., 1982), does not eliminate the ligand-induced decrease in the surface hydrophobicity.

Localization of the Estrogen- and Anti-Estrogen-Induced Decrease in Surface Hydrophobicity to the Steroid Binding Domain of the ER. On the basis of DNA sequence data for the ER, the steroid binding domain contains multiple hydrophobic amino acid residues and is predicted to be hydrophobic in nature (Green et al., 1986; Greene et al., 1986; Koike et al., 1987; Krust et al., 1986; White et al., 1987). Thus, the most obvious site for the loss of hydrophobic surface properties upon binding hormone would be within the steroid binding domain. We utilized the steroid binding domain (amino acids 241–595) of the human ER (originally from clone HEO; Green et al., 1986) overproduced in *E. coli* (Ahrens et al., 1990, 1992). This fragment of the ER (ER-C) contains only a few amino acids from the carboxyl end of the DNA binding domain as well as the entire D, E, and F domains. Aliquots containing the ER-C preparation were placed into the ATPP system described above. In the unsubstituted ATPP system, the unoccupied, E_2-occupied, and 4-OHT-occupied steroid binding domains all had similar K_{obs} values (Figure 1B, stippled bars). These same three forms of ER were then partitioned into the ATPP system with 10–20 μM PEG–palmitate present. As can be seen from Figure 1B (solid bars), the unoccupied ER-C moved dramatically into the upper phase. Occupation of the protein with E_2 or 4-OHT led to drastically decreased K_{obs} values in the PEG–palmitate system. Thus, the E_2- and 4-OHT-induced conformational changes that were determined by decreases in surface hydrophobicity described in Figure 1A were localized to the steroid binding domain of the ER. Although there was an observable difference in the K_{obs} values between the E_2- and 4-OHT-occupied ER forms in PEG–palmitate ($P < 0.1$ by Student's t test), this difference was already present in the unsubstituted phase system. This suggests minimal hydrophobic surface differences between the E_2- and 4-OHT-occupied ER complexes.

Characterization of the ER-C Steroid Binding Domain. To ensure that the overproduced steroid binding domain utilized in the ATPP systems had the same biophysical characteristics as the full-length ER and was therefore functionally normal, saturation binding analysis for ^{3}H-E_2 binding to ER-C at 4 °C was performed. The data were transformed by the method of Scatchard (1949), and a single high-affinity binding site for ^{3}H-E_2 was observed. The dissociation binding constant (K_d = 0.28 ± 0.01 nM) was estimated using a nonlinear reiterative fit by the computer program LIGAND (Munson & Rodbard, 1980). The amount of ^{3}H-E_2 binding activity in this preparation represents 11 pmol of steroid binding domain/mg of protein. For rat uterine cytosol at 3 uteri/mL, we routinely work with about 1 pmol of ER/mg of protein. Other than the high-affinity site, data from other experiments indicated that there may be a second, very low affinity binding site present in the ER-C preparation (Ahrens et al., 1990, 1992), on the order of 1 μM. However, this would not affect our ATPP results because the final ligand concentration used for the studies summarized in Figure 1B was 5 nM. At 5 nM ligand, virtually none of the low-affinity sites would be occupied.

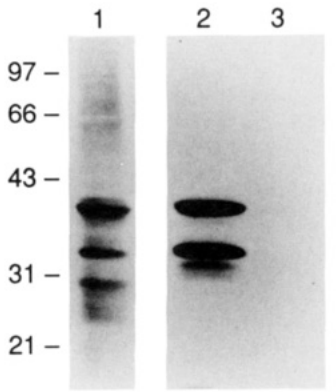

FIGURE 2: Determination of the size of the ER-C protein using immunoblotting and fluorography of affinity-labeled samples. The proteins in an aliquot of the unoccupied ER-C preparation were separated by SDS–PAGE (10% acrylamide), transferred to nitrocellulose, and immunostained with antibody ER712 (1:2500), lane 1. An aliquot of the overexpressed steroid binding domain was incubated with 25 nM ^{3}H-TAMZ, lane 2, or with 25 nM ^{3}H-TAMZ plus 1 μM DES as competitor, lane 3, at 4 °C for 1.5 h, and separated on the same SDS–PAGE as the sample in lane 1. Lanes 2 and 3 were fixed, treated with ENHANCE, dried and placed on film at −70 °C for 7 days. The locations at which the molecular mass markers migrated are shown on the left in kilodaltons.

The sizes of the overexpressed steroid binding domain fragments in the ER-C preparation were determined by immunoblotting with anti-ER antibody ER712 (Furlow et al., 1990) as shown in Figure 2, lane 1. At least three major fragments at 41, 36, and 29 kDa as well as other minor fragments were observed. This blot was intentionally exposed to substrate for a long period of time to show all possible bands. The most intense band near 41 kDa represents the predicted size of the intact ER-C protein. To determine which of these fragments bound hormone, an aliquot of the steroid binding domain preparation was incubated with 25 nM ^{3}H-TAMZ at 4 °C (Figure 2, lane 2). To control for nonspecific binding, 1 μM DES was added to an identical aliquot containing 25 nM ^{3}H-TAMZ (Figure 2, lane 3). Lanes 2 and 3 in Figure 2 show that ^{3}H-TAMZ is bound to the 41- and 36-kDa fragments and that there is virtually no nonspecific binding of ^{3}H-TAMZ in these bacterial extracts. Thus, the steroid binding domain overproduced in *E. coli* comprised two major fragments that could bind hormone, similar to previously reported results using ER-C prepared slightly differently (Ahrens et al., 1990, 1992). The different partition coefficients observed using affinity partitioning were therefore unlikely to be due to differences in the size of the overexpressed steroid binding domain.

Since the surface properties of a protein can be altered if it forms higher molecular weight complexes with other proteins, we investigated whether the change in hydrophobic surface properties of the ER-C protein upon binding E_2 could represent a change in the oligomeric state of the steroid binding domain. We analyzed the elution profile of the unoccupied (Figure 3A) or the E_2-occupied (Figure 3B) ER-C protein on a Sephacryl S200 gel filtration column. Two peaks of ^{3}H-E_2 binding were identified for both the unoccupied and E_2-occupied steroid binding domains. The peak in the void volume was relatively sharp for both ER forms and contained 45–60% of the binding activity for the ^{3}H-E_2-occupied ER and 62–84% of the binding activity of the unoccupied ER. The remainder of the ^{3}H-E_2 binding activity was found in the second peak, which was a broader peak ranging from about 31 to 80 kDa

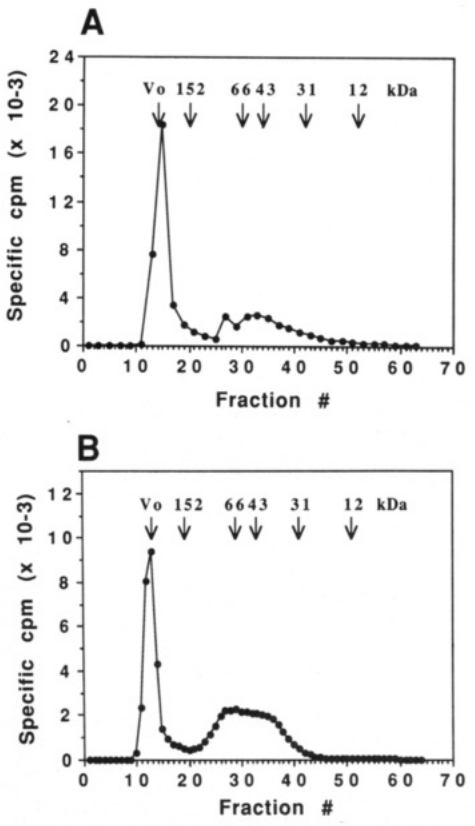

FIGURE 3: Gel filtration chromatography of unoccupied and E_2-occupied ER-C preparations. (A) The proteins in an aliquot of an unoccupied ER-C preparation were separated on a Sephacryl S200 gel filtration column. (B) An aliquot of the ER-C preparation was incubated with 5 nM ^{3}H-E_2 for 1.5 h at 4 °C, and the proteins were separated on the Sephacryl S200 column. Nonspecific binding was determined by an independent separation of the proteins in an aliquot of the ER-C preparation incubated with 5 nM ^{3}H-E_2 plus 1 μM DES. The peak fractions in which the protein standards used to calibrate the column eluted are indicated by arrows at the top with the molecular mass (in kilodaltons) for each protein shown above the arrows. These plots are representative of two independent samples run over the column. The percent of the total binding activity recovered in the void volume for the unoccupied ER-C ranged from 62 to 84% and for the E_2-occupied ER-C ranged from 45 to 60%.

in size. At least 50% of the steroid binding domain was clearly in a large oligomeric complex of some kind, eluting in the void volume, but there was only a small difference in the elution profiles when the receptor was unoccupied or E_2-occupied. The small difference in the amount of steroid binding domain in the void volume could not account for the large difference observed between the unoccupied and the E_2-occupied steroid binding domains with ATPP in PEG–palmitate. We do not know whether the ER-C protein eluting in the void volume represents the same protein complexes for the unoccupied and E_2-occupied preparations. The exact nature of this large oligomeric complex of the overexpressed steroid binding domain from bacterial extracts is unknown at this time.

The Steroid Binding Domain of the Rat Uterine Cytosolic ER Generated by Trypsinization Shows Behavior Similar to the ER-C Protein. To confirm the results obtained using the *E. coli*-produced steroid binding domain of the human ER, we generated the steroid binding domain from the rat uterine cytosolic ER using trypsin treatment. Figure 4 shows a fluorograph of ^{3}H-TAMZ-labeled rat uterine ER. If the cytosolic ER was occupied with ^{3}H-TAMZ (lane 1, O) or the unoccupied ER was heated and then occupied with ^{3}H-TAMZ (lane 5, UH), the full-length 66-kDa ER represents the major fragment observed following separation by SDS–PAGE.

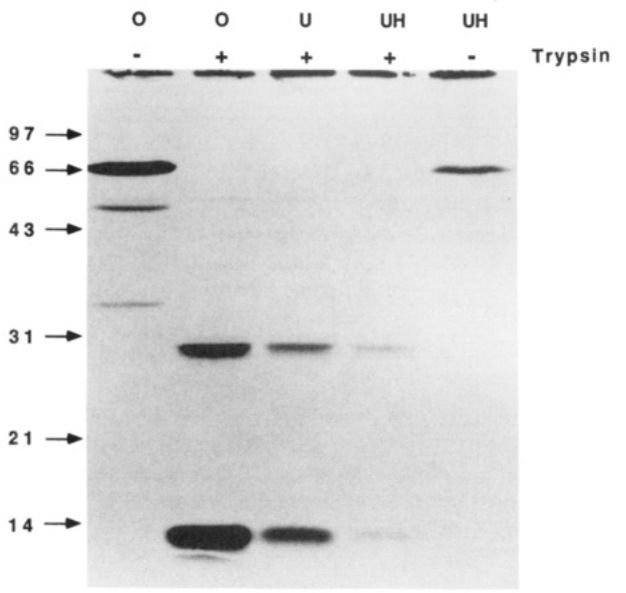

FIGURE 4: Fluorograph following SDS–PAGE of ^{3}H-TAMZ-labeled rat uterine cytosolic ER before and after trypsin treatment. Lane 1 contained rat uterine cytosolic ER incubated with 15 nM ^{3}H-TAMZ for 1.5 h at 4 °C. Lane 2 contained ^{3}H-TAMZ-occupied ER treated with trypsin (5 μg/mL) for 1 h at 4 °C. Lane 3 contained unoccupied ER treated with trypsin for 1 h at 4 °C and then occupied with 15 nM ^{3}H-TAMZ for 1.5 h at 4 °C. Lane 4 contained unoccupied ER that was heated at 30 °C for 45 min, treated with trypsin for 1 h at 4 °C, and then occupied with 15 nM ^{3}H-TAMZ. Lane 5 contained unoccupied ER that was heated at 30 °C for 45 min and then occupied with 15 nM ^{3}H-TAMZ. All trypsin treatment was stopped at the end of 1 h using a mixture of aprotinin and PMSF. The samples were boiled in sample buffer and separated by SDS–PAGE (12% acrylamide). The locations at which the molecular mass markers migrated are shown on the left in kilodaltons.

Small amounts of the previously reported fragments at 50 and 37 kDa were also observed (Katzenellenbogen et al., 1987). The intensities of the bands for the unoccupied–heated ER were less than for the ^{3}H-TAMZ-occupied ER because heating the unoccupied ER resulted in the loss of approximately 50% of the ligand binding ability (Hansen & Gorski, 1989, data not shown). Immunoblotting studies indicated that the loss of ER binding activity upon heating the unoccupied ER did not represent a loss of the 66-kDa ER protein (data not shown). If the cytosolic ER was occupied with ^{3}H-TAMZ and treated with trypsin (5 μg/mL) at 4 °C for 1 h and the fragments were separated by SDS–PAGE (lane 2, O), two labeled fragments were observed. The 28-kDa fragment represents the intact steroid binding domain, and the smaller 10-kDa fragment represents a segment of the steroid binding domain (Harlow et al., 1989; Ratajczak et al., 1989; Reese & Katzenellenbogen, 1991). Interestingly, if the unoccupied (lane 3, U) or the unoccupied–heated (lane 4, UH) ER was trypsinized for 1 h at 4 °C, protease inhibitors were added, and then it was occupied with ^{3}H-TAMZ for 1.5 h at 4 °C, the same 28- and 10-kDa fragments were generated. The only difference between the three trypsinized forms of the ER (O, U, UH) was in the intensity of the signals and not in the pattern of fragments generated. Thus, trypsin treatment generated the steroid binding domain, and there was no difference in the size of the fragments produced for the different ER forms.

The various forms of the trypsinized cytosolic ER were partitioned with and without 10–20 μM PEG–palmitate present, as shown in Figure 5. The K_{obs} values for all the various forms of the ER, with and without hormone or antihormone or after heating in the unsubstituted ATPP system,

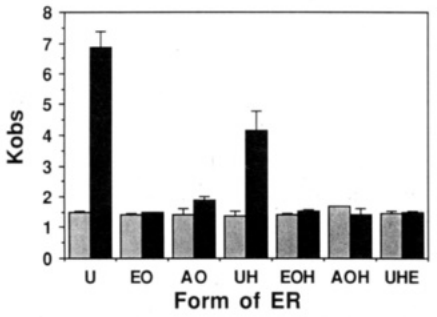

FIGURE 5: Partition coefficients for several forms of the trypsinized rat uterine cytosolic ER in the absence (stippled bars) or the presence (solid bars) of PEG–palmitate. The various receptor forms were the same as in Figure 1A with the addition of UHE, unoccupied ER heated to 30 °C for 45 min, and then occupied with ^{3}H-E_2 before addition of trypsin. Each receptor form was treated with 5 μg/mL trypsin for 1 h at 4 °C, followed by the addition of the protease inhibitors aprotinin and PMSF immediately prior to partitioning. K_{obs} values represent the mean ± SEM for two to four separate determinations, and the percent recoveries of the ER from the phase systems ranged from 31 to 81%.

were equivalent at about 1.5. Thus, removal of the amino terminus and/or the DNA binding domain dramatically alters the partitioning behavior in the unsubstituted system compared to the various full-length ER forms (Figure 1A), eliminating the heat- and ligand-dependent differences in this system. The unoccupied steroid binding domain (U, solid bar) generated by trypsin treatment showed a preference for the upper phase when PEG–palmitate was present, whereas the E_2-occupied (EO, solid bar) and 4-OHT-occupied (AO, solid bar) trypsin-generated steroid binding domains show no significant change in their partitioning behavior in the PEG–palmitate system as compared to the unsubstituted ATPP system. If these forms of the ER were heated before trypsin treatment, effects similar to those seen for the nonheated forms were observed. The unoccupied–heated steroid binding domain generated by trypsin preferred the PEG–palmitate-rich phase whereas the E_2-occupied–heated and 4-OHT-occupied–heated forms did not. To confirm that the unoccupied–heated ER could still respond to hormone appropriately, we added ^{3}H-E_2 to the unoccupied–heated ER and then trypsinized and partitioned this as the unoccupied–heated/E_2–occupied ER form (UHE). When hormone was added back to the unoccupied–heated ER, the hydrophobic surface properties measured by the increased affinity for the PEG–palmitate-rich upper phase disappeared. Thus, the effects of heat on ER structure are independent of the hormone-induced decrease in surface hydrophobicity. These data confirm that the steroid binding domain of the ER undergoes a conformational change upon binding estrogen or anti-estrogen as measured by a decrease in surface hydrophobicity.

Discussion

Upon binding estrogen, the ER has been proposed to undergo an as yet unidentified alteration in structure that leads to the transcriptional activation of estrogen-responsive genes. Our laboratory has previously shown that upon binding E_2 the ER undergoes a conformational change characterized by a significant decrease in surface hydrophobicity (Hansen & Gorski, 1986). Another laboratory has also suggested that hydrophobic properties of the ER may play a role in ER action (Hutchens et al., 1987a,b, 1990). In our current study, we show that a decrease in surface hydrophobicity upon binding E_2 is an inherent property of the steroid binding domain of the ER and is independent of any heat-induced conformational transitions. We propose that this decrease in surface hydrophobicity is an early and necessary step leading to a transcriptionally active ER.

It was previously hypothesized that the estrogen-induced decrease in surface hydrophobicity might involve the steroid binding domain (Hansen & Gorski, 1986). The steroid binding domain has been reported to fold into the globular mero-receptor (Sherman, 1984), and sequence data support the highly hydrophobic nature of the steroid binding domain. Using two independent sources of the steroid binding domain (*E. coli*-produced ER-C containing the human steroid binding domain and trypsin treatment of the rat uterine cytosolic ER), we demonstrated that the unoccupied steroid binding domain, when separated from the remainder of the ER, has hydrophobic surface properties as measured by a 3–5-fold increased affinity for the PEG–palmitate-rich upper phase (Figures 1B and 5). The unoccupied full-length rat uterine cytosolic ER showed a 2.7-fold increase in the K_{obs} value in the PEG–palmitate system versus the unsubstituted system whereas the *E. coli*-produced human steroid binding domain showed a 5.4-fold increase and the trypsinized rat uterine cytosolic ER showed a 4.6-fold increase. The difference in the fold increase between the steroid binding domain and the full-length ER probably indicates that the other domains of the full-length ER contribute to the surface properties of the ER as measured by partitioning. Clearly, the unoccupied steroid binding domain must be a dominant surface feature of the ER as demonstrated by the 3–5-fold increased affinity for the PEG–palmitate-rich upper phase. Upon binding estrogen, the steroid binding domain lost this hydrophobic surface property and showed little or no preference for the PEG–palmitate phase.

This estrogen-induced decrease in surface hydrophobicity appears to be an inherent property of the steroid binding domain of the ER and not due to other proteins bound to the ER. Although a significant proportion of the overexpressed steroid binding domain was in some form of oligomeric state (Figure 3), there was little difference in the elution pattern from the S200 gel filtration column when the steroid binding domain was unoccupied compared to E_2-occupied. Yet there was a significant decrease in surface hydrophobicity as measured by affinity partitioning. Similarily, in the completely different protein environment of rat uterine cytosol, we saw the same decrease in surface hydrophobicity of the trypsin-generated steroid binding domain upon binding E_2. If the unoccupied rat uterine ER was heated, trypsinized, and then subjected to affinity partitioning, we saw a similar preference for the PEG–palmitate-rich phase as when unoccupied–nonheated trypsinized ER was used, although the effect was somewhat blunted: 2.7-fold for the unoccupied–heated ER compared to 4.5-fold for the unoccupied–nonheated ER.

The observed decrease in hydrophobicity upon binding estrogen was shown to be independent of any heat-induced conformational changes. If the ER was occupied with E_2 and then heated, or the unoccupied ER was heated and then occupied with E_2, the hydrophobic surface properties were lost, which was the same as for the nonheated E_2-occupied form of the ER. This also excluded the possibility that hsp90 was involved in the estrogen-induced conformational change measured by affinity partitioning, because heating the unoccupied ER resulted in complete dissociation of hsp90 as measured by sucrose density gradient (data not shown). The ER without hsp90 still showed hydrophobic surface properties (Figures 1A and 5) and was able to respond to E_2 with a decrease in the surface hydrophobicity. These results suggest that the surface hydrophobicity is an inherent property of the unoccupied steroid binding domain and is not due to the

surface properties of other proteins bound to the ER. In addition, ligand binding was shown to be the main factor responsible for a decrease in surface hydrophobicity.

Since the anti-estrogen–ER complex does not lead to transcriptional activation of an estrogen-responsive gene (Jordan & Murphy, 1990; Nelson et al., 1988), we hypothesized that the 4-OHT–ER complex might retain some of the hydrophobic surface properties of the unoccupied ER. Surprisingly, the 4-OHT-occupied steroid binding domain showed a loss of surface hydrophobicity similar to the E_2-occupied complex. Thus, the difference between the estrogen–ER and anti-estrogen–ER complexes must be in some physical property other than surface hydrophobicity. The difference in the conformation of the two complexes could represent changes in surface charge or simply in surface shape. These differences could be important for interaction with other proteins necessary to build a functional transcription complex. Alternatively, our current assay system for measuring hydrophobic changes in the surface properties of the receptor may not be optimized to detect a subtle but significant difference in hydrophobicity between the E_2 and 4-OHT–ER complexes.

A previous study on the partitioning behavior of the anti-estrogen–ER complex indicated that it behaved more like the unoccupied ER than the estrogen–ER complex in an unsubstituted ATPP system (Hansen & Gorski, 1986). We observed a significant difference in the K_{obs} values for the E_2-occupied and the 4-OHT-occupied ERs in the unsubstituted ATPP system using the full-length rat uterine cytosolic ER (Figure 1A). Interestingly, when the steroid binding domain was partitioned in the unsubstituted ATPP system (Figures 1B and 5), the difference between the K_{obs} values for the E_2- and 4-OHT-occupied ERs disappeared, suggesting that the amino-terminal or DNA binding domains may be responsible for this difference in K_{obs} values. The decreased K_{obs} values for all three heated ER forms were not observed after trypsinization, suggesting that the amino-terminal and/or DNA binding domains are affected during heat-induced transformation (Grody et al., 1982), which must therefore involve more than just the loss of hsp90.

If estrogen or anti-estrogen is added to a cell containing unoccupied ER, the first observable change in ER behavior is that it becomes tightly held in the nucleus, requiring high salt (0.4–0.6 M KCl) for extraction. The nature and significance of tight nuclear binding remain poorly understood. We propose that the decreased hydrophobicity of the steroid binding domain upon binding ligand may be the important receptor modification that leads to tight nuclear binding within the cell. Tight nuclear binding involves more than just binding of ER to DNA because the unoccupied ER binds DNA with an affinity similar to the E_2- or 4-OHT-occupied ERs (Curtis & Korach, 1990, 1991; Klein-Hitpass et al., 1989; Murdoch et al., 1990). Further data supporting a role for the interaction of steroid hormone receptors with nuclear components other than just DNA include the observation that the ER dissociates completely from DNA at a slightly lower salt concentration (0.3 M KCl) than is necessary for extraction from the nucleus (Murdoch & Gorski, 1991; Murdoch et al., 1991; Skafar & Notides, 1985). Also, at elevated temperatures (25 °C), the association and dissociation rates of steroid receptor binding to DNA are very fast (Schauer et al., 1989). Thus, other interactions must hold steroid receptors tightly in the nucleus. Another component of tight nuclear binding may involve steroid receptor interaction with chromatin proteins in the immediate environment of the receptor bound to DNA. ER interaction with other chromatin proteins has been previously proposed (Getzenberg et al., 1990; Nelson et al., 1986; Spelsberg et al., 1983, 1988). These proteins could include histones, other transcription factors, proteins of the nuclear matrix, or others (Gross & Garrard, 1988; Johnson & McKnight, 1989). The unoccupied ER upon binding to DNA will have a large hydrophobic steroid binding domain in proximity to any proteins near this site. If the other proteins in the environment have hydrophilic or charged surfaces, the unoccupied steroid binding domain may not be able to form stable interactions with them (Nelson et al., 1989). When the steroid binding domain is occupied by E_2 or 4-OHT, this hydrophobic surface is buried, leaving a more hydrophilic surface capable of interacting more favorably with other hydrophilic chromatin proteins in the vicinity of the receptor bound to DNA. This added stabilizing interaction of the occupied steroid binding domain with other proteins, along with DNA binding, would allow the ER to become tightly bound within the nucleus, requiring high salt to break these interactions.

We propose here that the exposed hydrophobic surface of the unoccupied steroid binding domain of the ER could lead to unfavorable interactions with charged or hydrophilic proteins and thereby prevent receptor from becoming tightly bound to the nucleus until these hydrophobic residues are buried, following the binding of ligand. Tight nuclear binding is the first observed event following ligand binding and occurs for both E_2 and 4-OHT. Both E_2 and 4-OHT cause a decrease in surface hydrophobicity of the steroid binding domain of the ER as measured by ATPP and thus correlate with tight nuclear binding. Clearly, however, the most important part of signal transduction must be the final surface configuration of the receptor that exposes the transcriptional activation function by E_2 and prevents activation by 4-OHT.

We propose that the estrogen-induced change in the surface properties of the ER leads to the exposure of transcriptional activation functions that allow the ER to interact with other transcription factors or possibly allow sites on the ER to become available for covalent modification. We believe this conformational change is a critical step in hormone action.

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