A-Ring Nitro- and Amino-Substituted Estradiol Analogs Produce a Negative Cooperative or Noncooperative [^{3}H]Estradiol−Estrogen Receptor Binding Mechanism†

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ABSTRACT: We have investigated the relation between ligand structure and binding mechanism between the calf uterine estrogen receptor. A series of structurally altered estradiol analogs was used in which either an amino- or a nitro group had been added to the 2 or 4 position on the phenolic A-ring. The binding affinity of both amino analogs and the 4-nitro analog for the estrogen receptor was reduced relative to that of estradiol, as measured by competitive binding assay; the values were between 0.008% and 8% of estradiol's affinity. The slope of the displacement curve for the 4-nitro analog was also significantly different from that of estradiol ($p < 0.05$), indicating that the binding mechanism of these two ligands was different. The affinity of the 2-nitroestradiol ligand for the receptor was too low to be measured. The binding mechanism was then further investigated by measuring the Hill coefficient of [^{3}H]estradiol binding in the presence of the analog. The presence of a nitro group on C4 eliminated the positive cooperativity of the [^{3}H]estradiol−estrogen receptor interaction; the Hill coefficient of [^{3}H]estradiol binding in the presence of the analog was 0.99 compared with 1.7 for [^{3}H]estradiol alone. Most interestingly, the presence of an amino group on either C2 or C4 brought about a switch from a positive to a negative cooperative binding interaction; the Hill coefficients of [^{3}H]estradiol binding in the presence of the analogs were between 0.6 and 0.7. These results provide additional support for an induced-fit mechanism of ligand−estrogen receptor interactions.

The estrogen receptor is an enhancer binding transcription factor which can be activated by hormone. Considerable evidence indicates that activation of the estrogen receptor and other members of the nuclear receptor superfamily relies on the conformational changes brought about by hormone binding. Indeed, there is direct physical evidence for hormone-induced conformational changes in the thyroid hormone receptor by observed changes in the CD spectrum, though no comparable experiments have been published for the estrogen receptor at this time (Toney et al., 1993).

Estrogenic and antiestrogenic ligands make different contacts with the estrogen receptor; it has been suggested that their sites of interaction, although not identical, may overlap. They also provoke different biological responses, as measured by transcription activation (Reese & Katzenellenbogen, 1991; Pakdel & Katzenellenbogen, 1992; Danielian et al., 1993; Reese et al., 1992). It has also been shown that anti-estrogen-bound receptor can bind to DNA and induce structural changes in chromatin, yet still fail to activate transcription (Pham et al., 1991). These findings are consistent with studies on the physicochemical properties of ligand−receptor complexes, which indicate that the anti-estrogen-bound receptor has a different conformation than the estrogen-bound receptor (Ruh et al., 1990; Fritsch et al., 1992). Ligand-induced conformational changes may also affect the transcriptional properties of the estrogen receptor by shifting the relative proportions of monomeric and dimeric receptor (Ruh et al., 1990) as well as by affecting the conformation of the dimer itself (Schwartz & Skafar, 1993). Additional support for conformational changes of the receptor due to the presence of ligand comes from studies of the binding mechanism between different ligands and the receptor.

There is evidence that the active form of the estrogen receptor is a homodimer with one hormone binding site per monomer (Notides et al., 1985; Humar & Chambon, 1988) and that the estradiol−estrogen receptor binding interaction is positive cooperative (Weichman & Notides, 1977; Notides et al., 1981). Positive cooperative ligand binding is demonstrated by a concave-downward Scatchard plot and a Hill coefficient of 1.68 ± 0.21 (Notides et al., 1981). It is also known that different ligands produce distinct effects on the positive cooperative [^{3}H]estradiol−estrogen receptor binding interaction (Sasson, 1991). The weak agonists estriol and estrone, as well as the estrogenic antagonist transclomiphene, eliminate the positive cooperative binding of [^{3}H]estradiol to the receptor and cause the binding mechanism to become noncooperative (Sasson & Notides, 1982, 1983). For these ligands, a linear rather than convex Scatchard plot is observed, and the Hill coefficient is decreased from 1.61 ± 0.02 for estradiol to 1.04 ± 0.04 for estriol, 0.99 ± 0.03 for estrone (Sasson & Notides, 1983), and 1.10 ± 0.02 for clomiphene (Sasson & Notides, 1982). These studies show that certain ligands prevent the conformational changes necessary to the site−site interactions involved in the positive

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cooperative binding of estradiol (Sasson, 1991). In the presence of the estrogen antagonist 4-hydroxytamoxifen, complex [^{3}H]estradiol binding curves were obtained, and a Hill coefficient could not be measured (Sasson & Notides, 1988).

In a previous study done in this laboratory, A- and D-ring estradiol derivatives were tested for their effects on the positive cooperative estradiol−estrogen receptor binding interaction (Schwartz & Skafar, 1993). We were able to show that changes in the positive cooperative binding mechanism were related to subtle changes in the position of the functional group on the estradiol molecule. We also suggested that, in view of these and other data, the allosteric mechanism of the estrogen receptor coupld be best described in terms of an induced-fit model. In the present study, we used competition and saturation binding assays to test the effects of four additional estradiol analogs on the [^{3}H]-estradiol−estrogen receptor binding mechanism. In these analogs, either a nitro group or an amino group had been added to the 2- or 4-carbon on the A-ring of estradiol. We assessed the effects of these ligands on the cooperative estradiol−estrogen receptor binding interaction by using the changes in the shape of the Scatchard plot and Hill coefficient as a measure of the change in cooperativity. We found that the ability to compete with [^{3}H]estradiol for binding to the receptor was weakened and the positive cooperative binding of [^{3}H]estradiol to the estrogen receptor was perturbed. Data from recent experiments in another laboratory using these same ligands show that their ability to induce gene expression is also diminished (VanderKuur et al., 1991). We suggest that the perturbation in cooperativity induced by the presence of structurally altered ligand may be linked to alterations in transcriptional activation of specific genes. Finally, we describe the ability of a ligand to produce conformational changes in the estrogen receptor and activate transcription in terms of an induced-fit mechanism.

EXPERIMENTAL PROCEDURES

Materials

The 17β-[6,7-^{3}H]estradiol (64.1 Ci/mmol) was purchased from DuPont, NEN Products (Boston, MA). The estradiol analogs (2-nitroestradiol, 4-nitroestradiol, 2-aminoestradiol, and 4-aminoestradiol) were provided by Dr. S. C. Brooks, Biochemistry Department, School of Medicine, Wayne State University (Detroit, MI), and were synthesized as described in Werbin and Holloway (1956), Krachy and Gallagher (1957), Pickering and Werbin (1958), and Tomson and Horwitz (1959). Unlabeled ligands were stored in a nitrogen atmosphere and were kept in the dark for all studies. Phenylmethanesulfonyl fluoride (PMSF), dithiothreitol, ethylenediamintetraacetic acid (EDTA), Tris, and estradiol were from Sigma (St. Louis, MO); charcoal (Norit A) was from Fisher Scientific Co. (Fairlane, NJ); and Dextran T500 was from Pharmacia (Piscataway, NJ). The ammonium sulfate was from Schwarz/Mann (Cleveland, OH) and was ultrapure grade. All other chemicals were reagent grade.

Methods

Preparation of Estrogen Receptors. Preparation of the calf uterine cytosol has been previously described by Weichman and Notides (1977). Uterine cytosol was made 30% saturated with ammonium sulfate, allowed to sit on ice for up to 1 h, and centrifuged for 10 min at 27000*g*. After removal of the supernatant, the pellets were stored at −80 °C. For further use, receptor pellets were thawed on ice and dissolved in ice-cold TDE buffer: 40 mM Tris, 1 mM dithiothreitol, 0.1 mM EDTA, and 0.2 mM PMSF, pH 7.4 at room temperature.

Competitive Binding Assay of Estradiol and Estradiol Analogs. Competitive binding assays were carried out as described by Weichman and Notides (1977). Aliquots (200 μL each) of the dissolved ammonium sulfate fraction were incubated in duplicate for 3 h at 25 °C nM [^{3}H]estradiol plus 9×10^{-12} M to 9×10^{-6} M unlabeled competitor. Then, 100 μL of ice-cold Dextran-coated charcoal suspension (1% w/v charcoal plus 0.01% Dextran 500 in ice-cold TDE buffer) was added to each tube and incubated on ice for 10 min. Following centrifugation at 750*g* for 5 min at 4 °C, 100 μL aliquots were removed from the supernatant, and the bound [^{3}H]estradiol was measured by liquid scintillation counting in 3 mL of scintillation fluid. Nonspecific [^{3}H]estradiol binding was determined in a parallel incubation containing a 200-fold molar excess of unlabeled estradiol. The nonspecific binding value was always less than 5% of the total bound [^{3}H]estradiol.

Time To Reach Equilibrium in the Presence of Competitor. It has previously been determined that the estradiol binding to the estrogen receptor reaches equilibrium in 15−30 min and remains stable for 3 h at 25 °C (Sasson & Notides, 1983). The time for [^{3}H]estradiol binding to reach equilibrium in the presence of unlabeled competitor was measured. For each competitor, the concentration required to reduce the B_{max} of [^{3}H]estradiol binding to the estrogen receptor by 50% was used. Redissolved ammonium sulfate pellets and ligand mixtures were incubated at 25 °C for up to 3 h. During this time, 200 μL aliquots were successively removed at 0, 30, 60, 120, and 180 min. Free steroid and bound steroid were separated by Dextran-coated charcoal assay; radioactivity was measured by scintillation counting as above, and then corrected for nonspecific binding. The presence of 4-nitroestradiol, 2-aminoestradiol, or 4-aminoestradiol increased the time required for each equilibrium to 1, 2, or 3 h, respectively.

Binding of [^{3}H]Estradiol to the Estrogen Receptor in the Absence and Presence of a Constant Molar Excess of Competitor. To measure the effects of unlabeled competitor upon the equilibrium binding of [^{3}H]estradiol, the indicated ligand was added in a fixed molar ratio relative to each [^{3}H]-estradiol concentration. Twenty microlitor aliquots of each dilution were added to 200 μL aliquots of the resuspended ammonium sulfate fraction of calf uterine cytosol. This was performed in duplicate using siliconized test tubes. The final concentrations of [^{3}H]estradiol were between 0.25 and 20 nM. In a parallel set of incubations, a 200-fold excess of unlabeled estradiol was also added to measure nonspecific binding. Incubations were carried out at 25 °C for 1 h (4-nitroestradiol), 2 h (2-aminoestradiol), or 3 h (4-aminoestradiol). Ten minutes before the end of the incubation, a 50 μL aliquot was removed from each tube to determine the total [^{3}H]estradiol concentration. Then all tubes were placed on ice, and 100 μL of ice-cold, dextran-coated charcoal suspension was added to each tube, and the mixture was incubated for 10 min at 0 °C to adsorb the unbound estradiol and centrifuged at 750*g* for 5 min at 4 °C. One hundred

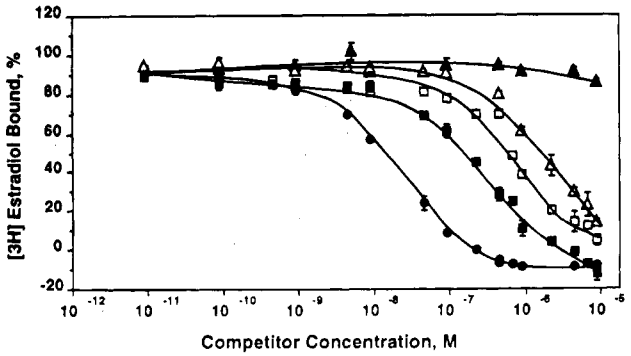

FIGURE 1: Competition between unlabeled competitor and [^{3}H]-estradiol for binding to the estrogen receptor. A saturating concentration of [^{3}H]estradiol was incubated with the indicated concentrations of unlabeled unlabeled estradiol (●), 2-nitroestradiol (▲), 4-nitroestradiol (△), 2-aminoestradiol (■), or 4-aminoestradiol (□), as described under Methods. These results are the averages ± SEM of four separate experiments.

microliter aliquots of the supernatant were removed, and the bound [^{3}H]estradiol was measured by liquid scintillation counting.

Stability Assay of the Estrogen Receptor. Two hundred microliter aliquots of unliganded receptor were incubated at 25 °C for the same length of time as the saturation binding assay, while incubating other aliquots of unliganded receptor for the same period of time at 0 °C. Each of the samples was then incubated with a saturating concentration of [^{3}H]-estradiol in the presence or absence of unlabeled competitor for 1 additional h at 0 °C, in order to measure specific binding. Only those experiments in which receptor inactivation, defined as the difference in specific binding between the aliquots incubated at 0 °C and those incubated at 25 °C, was 5% or less were used.

Data Analysis. Bound and free [^{3}H]estradiol were calculated using Lotus 1-2-3. These values were used in Lotus 1-2-3 to determine the B_{max} from the limiting slope of the Scatchard plot (Scatchard, 1949), and the Hill coefficient from the maximum slope of the Hill plot (Hill, 1910); these data were used for the insets of Figures 2−6. Data for bound and free ligand were further analyzed using Enzfitter (Elsevier Biosoft) which determines the B_{max} and the Hill coefficient by nonlinear regression analysis; these data were used in Table 2.

RESULTS

The ability of one ligand to influence the binding of another was examined by competition and saturation binding assay. Competition binding measures the affinity of an unlabeled ligand for the estrogen receptor compared with the affinity of [^{3}H]estradiol for the receptor. The relative binding affinities of the 2- and 4-amino-substituted estradiol ligands were 7.71% and 1.96%, respectively, of that for estradiol (Figure 1; Table 1). While the 4-nitro-substituted estradiol had a binding affinity of 0.83% relative to that of estradiol, the 2-nitroestradiol did not compete with [^{3}H]-estradiol for binding to the receptor (Figure 1; Table 1). Because of this undetectable affinity, further studies with this ligand could not be done. When analyzed by the F-test for parallelism, the slope of the displacement curve for 4-nitroestradiol was significantly different from that of estradiol ($p < 0.05$), indicating that the binding mechanism of the two ligands was different.

Table 1: Relative Binding Affinity[a]

ligand	relative binding affinity
4-nitroestradiol	0.0083
2-aminoestradiol	0.0771
4-aminoestradiol	0.0196
estradiol	1.0000

[a] A quantitative comparison of binding affinities relative to that of estradiol is shown for the three different estradiol analogs. This was done by determining the competitor concentrations required to cause a 50% inhibition of [^{3}H]estradiol binding, compared with the concentration of estradiol required to cause a 50% inhibition of [^{3}H]estradiol binding. These values were calculated from the data in Figure 1.

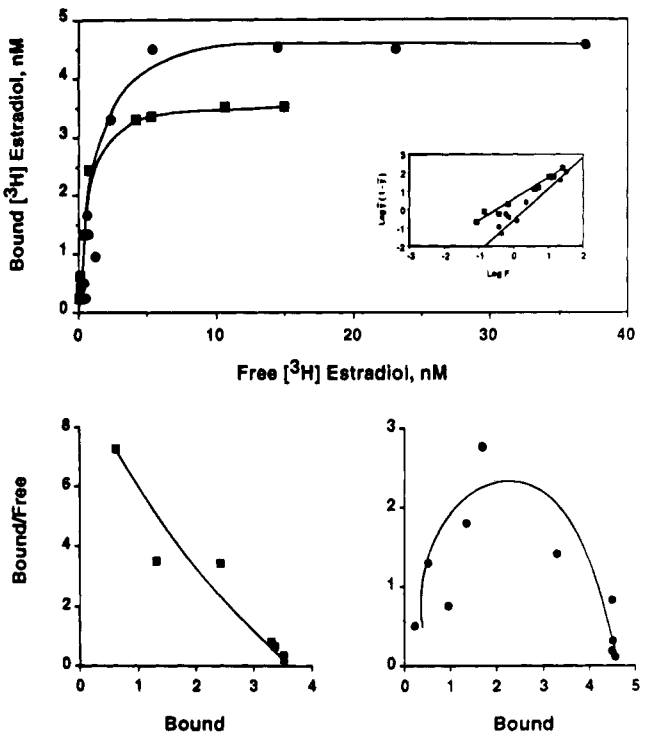

FIGURE 2: Saturation binding analysis of the binding of [^{3}H]-estradiol to the estrogen receptor in the presence of a constant molar excess of 4-nitroestradiol. The binding of [^{3}H]estradiol to the estrogen receptor was measured in the absence of competing ligand (●) and in the presence of 4-nitroestradiol (■), as described under Methods. The saturation binding curves (top), corresponding Hill plots (upper inset), and Scatchard plots in the absence (right bottom) and in the presence (left bottom) of 4-nitroestradiol are shown. The Hill coefficients of the data shown are 1.08 ± 0.11 in the presence of 4-nitroestradiol and 1.69 ± 0.01 for [^{3}H]estradiol alone. These data are representative of four independent experiments.

The ability of each ligand to influence the [^{3}H]estradiol−estrogen receptor interaction was examined using saturation binding analysis to determine the Hill coefficient (Hill, 1910) and the shape of the Scatchard plot (Scatchard, 1949). The calf uterine estrogen receptor bound [^{3}H]estradiol with a maximum Hill coefficient of 1.69 ± 0.02; the corresponding Scatchard plots were convex (Figures 2−4; Table 2). These observations are consistent with the positive cooperative binding mechanism first observed by Notides, Lerner, and Hamilton in 1981. Saturation binding of [^{3}H]estradiol to the estrogen receptor in the presence of unlabeled 4-nitroestradiol, 4-aminoestradiol, or 2-aminoestradiol was then performed, and the Hill coefficients were determined to be 0.99 ± 0.09, 0.69 ± 0.04, and 0.65 ± 0.08, respectively (Figures 2−4; Table 2). Moreover, in the presence of 4-nitroestradiol, a linear Scatchard plot was observed, rather than the convex

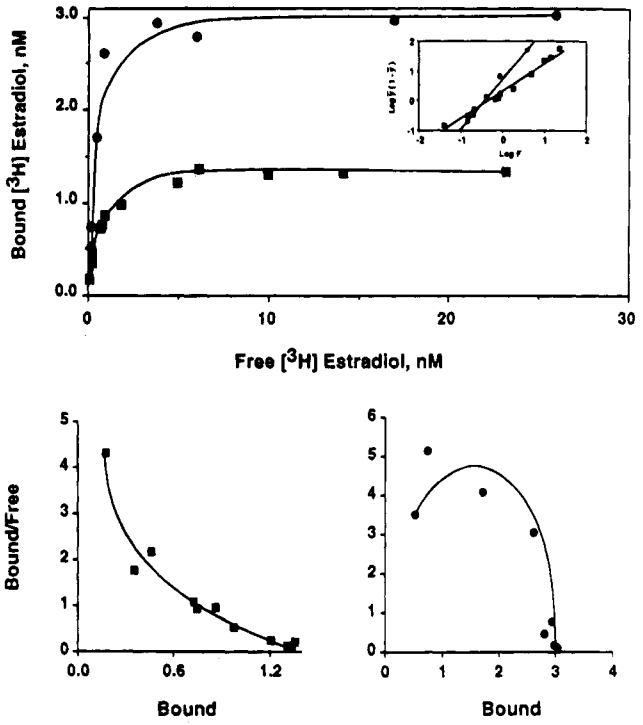

FIGURE 3: Saturation binding analysis of the binding of [^{3}H]-estradiol to the estrogen receptor in the presence of a constant molar excess of 4-aminoestradiol. The binding of [^{3}H]estradiol to the estrogen receptor was measured in the absence of competing ligand (●) or in the presence of 4-aminoestradiol (■), as described under Methods. The saturation binding curves (top), corresponding Hill plots (upper inset), and Scatchard plots in the absence (right bottom) and in the presence (left bottom) of 4-aminoestradiol are shown. The Hill coefficients of the data shown are 0.77 ± 0.01 in the presence of 4-aminoestradiol and 1.66 ± 0.21 for [^{3}H]estradiol alone.

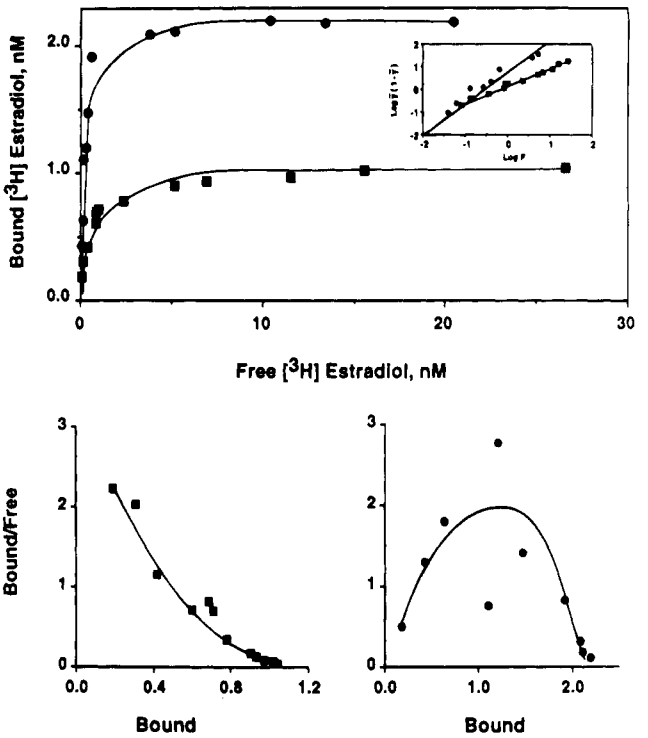

FIGURE 4: Saturation binding analysis of the binding of [^{3}H]-estradiol to the estrogen receptor in the presence of a constant molar excess of 2-aminoestradiol. The binding of [^{3}H]estradiol to the estrogen receptor was measured in the absence of competing ligand (●) and in the presence of 2-aminoestradiol (■), as described under Methods. The saturation binding curves (top), corresponding Hill plots (upper inset), and Scatchard plots in the absence (right bottom) and in the presence (left bottom) of 2-aminoestradiol are shown. The Hill coefficients of the data shown are 0.75 ± 0.07 in the presence of 2-aminoestradiol and 1.34 ± 0.13 for [^{3}H]estradiol alone.

plot observed in the presence of estradiol alone (Figure 2). In the presence of either 4-aminoestradiol or 2-aminoestradiol, a concave-upward Scatchard plot was observed instead of the convex plot observed with estradiol alone (Figures 3 and 4). It has previously been determined that the presence of equimolar unlabeled estradiol does not significantly alter the value of the Hill coefficient or the convexity of the Scatchard plot (Sasson & Notides, 1981; Schwartz & Skafar, 1993). These results are consistent with the idea that binding of the amino- and nitro-substituted estradiol analogs produced a noncooperative or negative cooperative binding interaction with the receptor.

Table 2: Cooperativity of [^{3}H]Estradiol and Unlabeled Competitor Binding to the Calf Uterine Estrogen Receptor[a]

ligand tested	average Hill coefficient
[^{3}H]estradiol	1.68 ± 0.05
+4-nitroestradiol	0.99 ± 0.09
+2-aminoestradiol	0.65 ± 0.08
+4-aminoestradiol	0.69 ± 0.04

[a] The Hill coefficients for [^{3}H]estradiol binding to the estrogen receptor in the absence and in the presence of unlabeled competitor are the averages ± SEM of 3−5 separate experiments. Receptor concentrations between 1.5 and 4.6 nM were used.

DISCUSSION

The calf uterine estrogen receptor exhibits the hallmarks of an allosteric protein (Notides et al., 1981, 1985; Skafar & Notides, 1987). One of these properties exhibited by the receptor is a positive cooperative binding mechanism for estradiol; estradiol binds with a Hill coefficient of 1.5−1.7 (Notides et al., 1981; Sasson & Notides, 1983). This positive cooperativity of the receptor means that the binding of the first molecule of ligand increases the affinity for the second molecule of ligand. Negative cooperativity—which is also observed in some allosteric proteins—means that binding the first molecule of ligand decreases the affinity for the second molecule of ligand. In general, cooperative ligand binding stems from and is a direct indicator of conformational changes in the protein (Wyman & Gill, 1990). Whether or not these types of changes are related to the differences in gene regulation brought about by the binding of different ligands to the estrogen receptor has not been directly shown, yet seems likely.

In this study, the effect of nitro- and amino-substituted estradiol ligands on the positive cooperative estradiol−estrogen receptor binding mechanism was analyzed. These ligands were selected because of their charge properties: the amino group adds a net positive charge; the nitro group has a positive nitrogen and a negative oxygen, so that it is polar, but its net charge is zero. By investigating the influence of structurally altered ligands on the binding mechanism of estradiol with the receptor, their ability to affect the conformation of the receptor could therefore be assessed. Moreover, studying the binding of the *combination* of estradiol with the altered analogs mimics an *in vivo* situation in which the exogenous compound would be present together with an estradiol background. The results showed that, in

the presence of 4-nitroestradiol, the positive cooperative binding of [^{3}H]estradiol to the receptor was eliminated. This was demonstrated by a linear Scatchard plot and a decrease in the Hill coefficient from 1.68 to 0.99 ± 0.09 (Figure 2, Table 2). The slope of the displacement curve for the 4-nitroestradiol was also significantly different from that of estradiol, indicating the two ligands bind by different mechanisms (Figure 1). Even more strikingly, the presence of either 4-aminoestradiol or 2-aminoestradiol brought about a clear switch from positive to negative cooperativity; the Scatchard plots became concave-upward, and the Hill coefficients decreased to 0.69 ± 0.04 and 0.65 ± 0.08, respectively (Figure 3 and 4). These findings show that while the positive cooperative binding of [^{3}H]estradiol to the receptor is eliminated by the 4-nitro-substituted ligand, the cooperativity becomes negative in the presence of either the 4- or the 2-amino-substituted analogs. There are other examples of ligands that convert the binding mechanism from positive cooperative to noncooperative, such as the anti-estrogen clomiphene and estradiol analogs whose A-ring hydroxyl has been moved from the 3 position (Sasson & Notides, 1982; Schwartz & Skafar, 1993). However, this is the first demonstration that the presence of a structurally altered estradiol analog can produce a *negative* cooperative binding mechanism.

At the concentrations of receptor used, 1.5−4.6 nM, the ammonium sulfate precipitated calf uterine estrogen receptor displays full cooperativity of [^{3}H]estradiol binding and sediments as a 5S species on sucrose density gradients, indicating it is in the dimeric state (Notides et al., 1985). The observation of negative cooperativity in the presence of either amino analog is additional evidence of a dimer. Note that a noncooperative binding mechanism, as was observed in the presence of the nitro analog, could be explained either by loss of dimerization or by loss of site−site interactions. We favor the idea that in the experiments presented here site−site interactions have been lost, since as mentioned above, the calf estrogen receptor forms a dimer under the conditions of these experiments.

Bulky anti-estrogens such as tamoxifen make contacts with the estrogen receptor that the endogenous ligand does not make (Pakdel & Katzenellenbogen, 1992). Since the ligands used in these studies are relatively small, it is likely that they align in the binding site more similarly to estradiol than to tamoxifen. However, it is possible that the group on the A-ring could affect the orientation of the molecule on the binding site, particularly since data indicate multiple polar or charged groups lie within or near the ligand binding site of the receptor (Pakdel & Katzenellenbogen, 1992).

It is reasonable to suggest that these charged groups on the receptor plays a role in the interactions with the modified ligands used in these studies, but the molecular mechanism by which the presence of charged amino group on the estradiol molecule produces a negative cooperative binding mechanism can only be discussed in general terms. First, a favorable electrostatic interaction between the charged ligand and an oppositely charged residue on the receptor could stabilize a conformation that was aberrant or incapable of further conformational change. Note that, since all the ligands tested in this report had a lower affinity for the receptor than did estradiol (Table 1), any favorable charge interaction must be overcompensated by unfavorable interactions and/or unfavorable changes in receptor conformation. Second, the charged group, if appropriately placed on or near the receptor surface, could compete for salt bridges commonly made at the protein interface between subunits, thereby weakening dimerization and/or site−site communication. Third, charge repulsion between ligands themselves, or between the ligand and a positively charged residue in the binding site of the receptor, might alter the binding site conformation and hinder site−site communication. Finally, the charged group could interfere with hydrophobic interactions in the binding site and hinder conformational changes. If the ligand binding sites on the subunits are close enough, charge−charge repulsion would provide the simplest explanation for the negative cooperative binding mechanism observed when positively charged ligands were present.

Previously, it has been shown that these same ligands have a lower affinity for the estrogen receptor from MCF-7 cells than does estradiol (Brooks et al., 1987). In the earlier work, the 2-nitro derivative had the lowest affinity for the receptor, while the other three analogs each had relative binding affinities of 0.12−0.16. Our values are lower than those reported previously, although we too found the lowest affinity for the 2-nitro derivative. The difference may be due to the temperature of incubation (25 °C vs 0 °C), the source of receptor (bovine uterus vs human MCF-7 cells), or the concentration of receptor used (1.5−4.6 nM vs unreported).

Results from studies on the ability of these nitro- and amino-substituted estradiol ligands to activate transcription in MCF-7 cells provide additional information on the role of ligand-induced conformational changes in gene expression. The progesterone receptor is an estrogen-responsive gene in MCF-7 cells. The 2-aminoestradiol is less effective than estradiol in inducing progesterone receptor synthesis; the 2-nitroestradiol and 4-aminoestradiol ligands are ineffective in inducing progesterone receptor synthesis (VanderKuur et al., 1991). Moreover, 4-nitroestradiol did actively induce progesterone receptor synthesis (VanderKuur et al., 1991). The reduction in gene expression by the amino-substituted ligands in the above studies correlates with the Hill coefficients less than 1 measured in the studies presented here. There results suggest that the perturbation in cooperatively brought about by the presence of structurally altered, charged ligands is related to the reduction in transcriptional activation observed for these same ligands.

Further support for the role that ligand-induced allosteric changes play in gene expression comes from studies of estrogen receptor mutants that display "decoupling". These mutants show only a modest reduction in their affinity for hormone, yet display a drastic reduction in hormone-induced transcriptional activity (Fawell et al., 1990; Danielian et al., 1993; Pakdel & Katzenellenbogen, 1992). These results are best explained by alterations in postligand binding events, such as dimerization, DNA binding, phosphorylation, or interaction with transcription factors. Related possibilities include unfavorable changes in receptor conformation, or loss of site−site interactions. In the *lac* repressor, for example, mutation of tyrosine-282 to phenylalanine produces a protein that retains its tetrameric structure, but had altered cooperativity of inducer binding (Chakerian & Matthews, 1991). No cooperativity was observed in the mutant estrogen receptors (Pakdel & Katzenellenbogen, 1992). However, at the concentrations of receptor used, less than 0.5 nM, none would be expected; the human estrogen receptor requires concentrations over 10 nM to display full positive cooper-

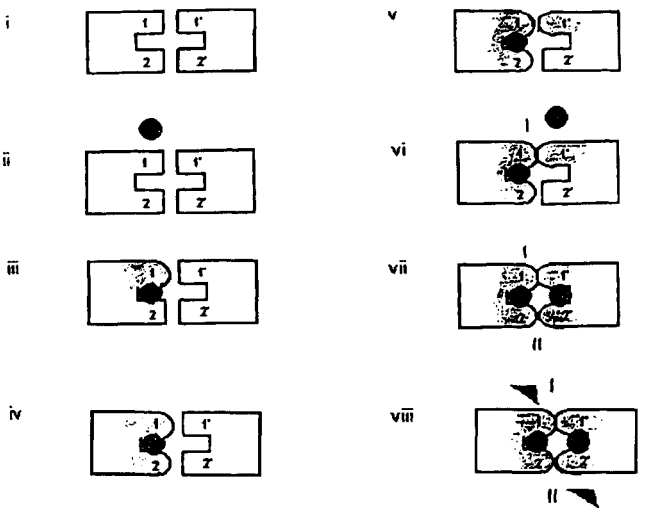

FIGURE 5: Speculative model for the induction of conformational change in the estrogen receptor by ligand. Conformational changes and protein–protein interactions are indicated by shading; the estradiol molecule, by a filled circle; and transcription factors, by filled triangles. We have depicted the unbound ER as a dimer for simplicity (i, ii). Low-affinity binding of the first molecule of ligand (iii) induces local conformational changes first at "1" and then at "2". The binding of the first molecule of ligand is low affinity because it requires changes at sites "1" and "2" for a good fit (iv). These changes transmit information to a neighboring site "1" on the adjacent subunit (v). These conformational changes also create the first transcription factor interaction surface, using sites on both subunits (I). Subunit–subunit contact may result in quaternary changes in the dimer which provide a route for communication to the neighboring subunit (vi). The second molecule of ligand binds with greater affinity because the conformational changes at 1′ has already occurred and only the conformational change at 2′ is needed. This change at 2′ permits a better fit and slower dissociation of the ligand (vii). As a result of these changes, the interface which has formed between 2 and 2′ creates a second surface for interaction with transcription factors or other proteins (II). The surfaces at 1–1′ and 2–2′ may interact with transcription factors in a gene-specific manner (viii). In this way, the completion of all conformational changes in this pathway, as for the estradiol-bound dimer, would produce a fully activated receptor. Partial or total inability to complete these conformational changes, which is suggested to occur for other receptor–ligand complexes, could produce an unactivated, partially activated, or even aberrant receptor. This, in turn, would reduce the ability of the complex to regulate gene expression.

ativity (Obourn et al., 1992). It would be interesting to test the binding mechanism of the mutant estrogen receptors at higher receptor concentrations to determine whether positive cooperativity has been altered.

Finally, a schematic model (Figure 5) is presented to illustrate the possible allosteric mechanism of the estrogen receptor and to show how the communication between binding sites across the subunit interface may alter the conformation of the receptor to a state that is capable of mediating transcriptional activation. This corresponds well with the model for ligand-induced conformational changes proposed by Koshland (Koshland et al., 1966). In this model, positive cooperativity arises from the sequential modulation of protein structure which would be complete, in the estrogen receptor, upon the binding of a second molecule of estradiol. We hypothesize that ligand binding starts a series of conformational changes which may be critical to the communication between ligand binding sites on the estrogen receptor. These ligand-induced conformational changes may enhance the interaction between dimerization surfaces and position receptor subunits for the site–site interaction necessary to the positive cooperative binding of a second molecule of ligand. The changes in conformation could also alter regions involved in transcription activation, such as surfaces for interaction with other transcription factors, i.e., TAF-2. Successfully completing the conformational changes would then lead to a protein with full biological activity. The inability to complete these conformational changes or the formation of an aberrant conformational state, due to either mutation in the receptor or altered ligand structure, would lead to partial or no activity.

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