Ligand-Mediated Modulation of Estrogen Receptor Conformation by Estradiol Analogs†

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ABSTRACT: The studies presented here show how changing the structure of the ligand can affect the conformation of the receptor. Five different estradiol analogs have been tested for binding to the calf uterine estrogen receptor. In three of the analogs the phenolic hydroxyl group had been moved from the 3 to the 1, 2, or 4 position on the A-ring (1-hydroxyestratrien-17β-ol, 2-hydroxyestratrien-17β-ol, or 4-hydroxyestratrien-17β-ol). In the remaining two analogs either the A- or the D-ring hydroxyl group had been removed altogether (estratrien-17β-ol or 3-hydroxyestratriene). Competition binding assay showed that the relative binding affinity for the estrogen receptor had been weakened by all changes in the structure of the ligand. Furthermore, the ligands in which either the 3β- or the 17β-hydroxyl group was missing produced nonparallel slopes in the linear portions of the displacement curves compared to that of estradiol; the ligands in which the phenolic hydroxyl had simply been moved around the A-ring, however, did not. These observations implied that the receptor binding mechanism used by the monohydroxyl ligands was different from that of estradiol. Saturation binding analysis showed that while the presence of any of the dihydroxyl ligands or that of estratrien-17β-ol decreased the positive cooperativity of the [^{3}H]estradiol–estrogen receptor interaction, the presence of the 3-hydroxyestratriene ligand increased it. These results suggest that both the binding mechanism and the affinity of the ligand for the receptor are exquisitely sensitive to the structure of the ligand.

The binding of estradiol to its receptor ultimately leads to the many observed biological responses to the hormone. One early step in this process is the production of conformational changes in the receptor by ligand binding. Previous studies have shown that two conformational states exist for the estrogen receptor which differ in their affinity for estradiol (Weichman & Notides, 1977). The formation of the high-affinity state is dependent on receptor dimerization and is characterized by positive cooperative estradiol binding, as demonstrated by a convex Scatchard plot and a Hill coefficient of 1.6 (Notides et al., 1981). Positive cooperativity is a type of self-regulation in which the binding of the first molecule of ligand to an oligomeric protein increases the affinity of the protein for additional molecules of ligand. The positive cooperative binding mechanism is then an indicator of conformational change in the protein. However, for the estrogen receptor, the relation between the structure of the ligand and its binding mechanism, that is, the presence and degree of positive cooperativity, has not been defined.

Structure–activity studies show that many different compounds bind to the estrogen receptor; however, binding affinity is not necessarily related to estrogenicity (Jordan & Tate, 1980). Numerous efforts have been aimed at correlating the position of the two hydroxyl groups on the estradiol molecule to the binding affinity and the conformational changes elicited. Early works proposed that binding affinity was dependent on the A-ring hydroxyl group (Duax et al., 1980; Jordan, 1984), while conformational changes, correct folding, and stabilization of the dimeric receptor form were dependent on the D-ring hydroxyl (Vedeckis et al., 1980; Duax et al., 1980; Jordan & Murphy, 1990). Recent findings suggest that this is an oversimplification. For example, although the phenolic hydroxyl group at C3 is extremely important for high-affinity binding to the estrogen receptor, its absence does not preclude binding or biologic activity. Estratrien-17β-ol, which lacks the 3-hydroxyl group, has a measurable binding affinity for the receptor and is able to induce progesterone receptor (Brooks et al., 1987). 3-Hydroxyestratriene, which lacks the 17β-hydroxyl group, has a higher affinity for the receptor and can also induce progesterone receptor (Brooks et al., 1987). On the basis of these studies, the contributions made by each of these functional groups cannot be distinguished.

The weak estrogen agonists estriol and estrone, when tested at 25 °C, show reduced positive cooperativity compared with that of estradiol, with Hill coefficients of 1.04, 0.99, and 1.61, respectively (Sasson & Notides, 1983). This suggests that estriol and estrone fail to induce the conformational changes which are induced by estradiol, and this correlates with their weaker biological activity.

Antiestrogens, such as tamoxifen, clomiphene, and ICI 164,384, have been suggested to inhibit conformational changes in the receptor necessary to transformation (Baulieu, 1987; Weatherill et al., 1988), dimerization (Fawell et al., 1990), or activation (Webster et al., 1988; Sasson, 1991). The bulky side chains present in these antiestrogens have been proposed to sterically hinder the receptor's conformational changes. One would therefore predict that the positive cooperative interaction induced by estrodiol would be inhibited by these antagonists. This is observed for clomiphene antiestrogens (Sasson & Notides, 1982). In contrast, when the binding mechanism of [^{3}H]-4-hydroxytamoxifen to the estrogen receptor was investigated by saturation binding analysis, a positive cooperative interaction, similar to that of estradiol, was observed (Sasson & Notides, 1988). However, when both ligands were present simultaneously, differences in their binding mechanisms became apparent. Collectively,

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these observations show that studies on the binding mechanism may be useful in understanding the biological activity of a ligand.

In this work we analyze the binding mechanism of five estradiol derivatives to the calf uterine estrogen receptor by competitive and saturation binding assay. The crystallographic conformations for three of these estradiol derivatives, in which the phenolic hydroxyl group has been moved around the A-ring, were recently determined (Palomino et al., 1990). The binding affinities and effects on nuclear binding and gene regulation of these and two monohydroxyl estradiol derivatives, in which either the A- or the D-ring hydroxyl group had been removed altogether, have also been determined (Pilat et al., 1993; VanderKuur et al., 1993). Although the functional group position, steroid conformation, relative binding affinity, and effects on gene expression were measured and correlated, the binding mechanism was not investigated. By examining the binding mechanism of these structurally altered ligands for the estrogen receptor, the studies presented here attempt to bridge the gap between structure and function for one group of steroids.

EXPERIMENTAL PROCEDURES

Materials

The 17β-[6,7-^{3}H]estradiol (64.1 Ci/mmol) was purchased from Du Pont, NEN Products (Boston, MA). The estradiol analogs (3-hydroxyestratriene, estratrien-17β-ol, and 1-, 2-, and 4-hydroxyestratrien-17β-ol) were provided by Dr. S. C. Brooks, Biochemistry Department, School of Medicine, Wayne State University (Detroit, MI). Their synthesis is described in Horwitz et al. (1986) and Palomino et al. (1990). The phenylmethanesulfonyl fluoride (PMSF), dithiothreitol, ethylenediaminetetraacetic acid (EDTA), Tris, and estradiol were from Sigma (St. Louis, MO); charcoal (Norit A) was from Fisher Scientific Co. (Fairlawn, 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 buffer: 40 mM Tris, 1 mM dithiothreitol, 0.1 mM EDTA, and 0.2 mM PMSF, pH 7.4, at 25 °C (TDE buffer).

Competitive Binding Assay of Estradiol and Estradiol Analogs. Aliquots (200 μL each) of the dissolved ammonium sulfate fraction were incubated in duplicate for 3 h at 25 °C with 10 nM [^{3}H]estradiol plus 9×10^{-12} 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 by parallel incubation of mixtures 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 to reach equilibrium with the receptor in the presence of unlabeled competitor was determined. For each competitor, the concentration required to reduce the B_{max} of [^{3}H]estradiol binding to the estrogen receptor by 50% was used. Cytosol–ligand mixtures were allowed to incubate 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 and bound steroid were separated by Dextran-coated charcoal assay, assessed for radioactivity by scintillation counting as above, and then corrected for nonspecific binding. The presence of 1-, 2-, or 4-hydroxyestratrien-17β-ol did not significantly prolong the time required for the estrogen receptor to reach equilibrium binding with labeled estradiol. The presence of estratrien-17β-ol or 3-hydroxyestratriene did prolong the time to reach equilibrium to 1 or 2 h, respectively.

Binding of [^{3}H]Estradiol to the Estrogen Receptor in the Absence and in the 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. The stock solutions were in 10% ethanol (v/v) in TDE buffer. Twenty-microliter aliquots of each dilution were added to 200-μL aliquots of the ammonium sulfate fraction of the resuspended calf uterine cytosol. This was performed in duplicate using siliconized test tubes. The final concentration of [^{3}H]estradiol was between 0.25 and 20 nM. A 200-fold excess of unlabeled estradiol was added together with the [^{3}H]estradiol, the [^{3}H]estradiol plus unlabeled competitor, or the [^{3}H]estradiol plus equimolar estradiol samples containing the receptor to determine nonspecific binding. 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; the mixtures were 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-microliter samples of the supernatant were removed, and the bound [^{3}H]-estradiol was measured.

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 other aliquots of unliganded receptor were incubated 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 absence and in the presence of unlabeled competitor for 1 additional hour at 0 °C, in order to determine specific binding. Only those experiments in which receptor inactivation 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 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 II.

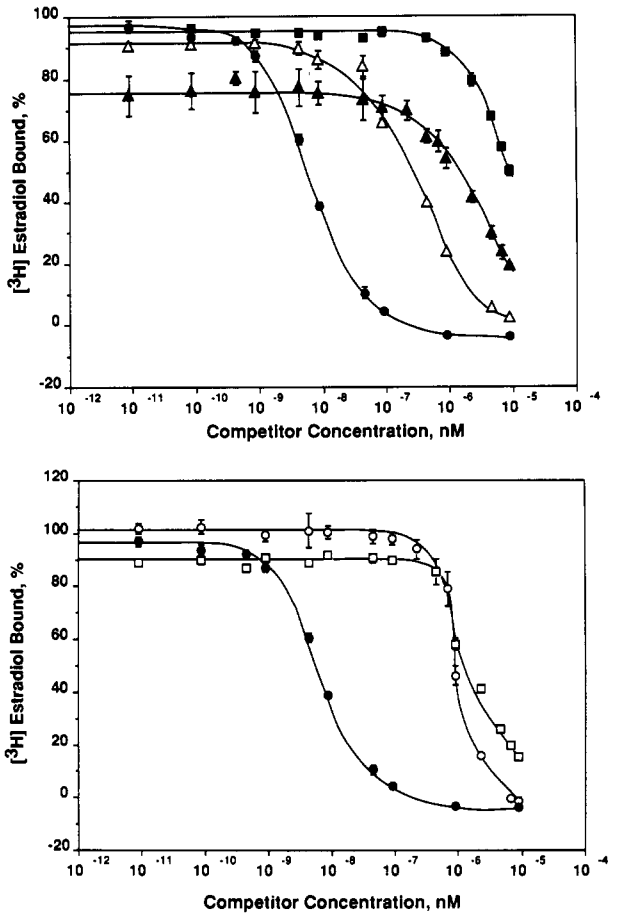

FIGURE 1: Competition between unlabeled competitor and [3H]-estradiol for binding to the estrogen receptor. [3H]Estradiol (10 nM) was incubated with the indicated concentrations of unlabeled estradiol (●), 1-hydroxyestratrien-17β-ol (■), 2-hydroxyestratrien-17β-ol (△), or 4-hydroxyestratrien-17β (▲) (top) and with unlabeled estradiol (●); 3-hydroxyestratriene (○); or estratrien-17β-ol (□) (bottom), as described in Methods. These results are the averages ± SEM of four independent experiments.

Table I: Relative Binding Affinities[a]

ligand	rel binding affinity
1-hydroxyestratrien-17β-ol	0.0022
2-hydroxyestratrien-17β-ol	0.0874
4-hydroxyestratrien-17β-ol	0.0132
estratrien-17β-ol	0.0151
3-hydroxyestratriene	0.0248
estradiol	1.0000

[a] A quantitative comparison of binding affinities relative to that of estradiol is shown for the five different estratriene derivatives. They were calculated by determining the competitor concentrations required to cause a 50% inhibition of [3H]estradiol binding and comparing that concentration with the concentration of unlabeled estradiol required to reduce specific binding by 50%. These values were determined from the data in Figure 1.

RESULTS

The ability of one ligand to influence the binding of another was examined by competition and saturation binding assay. Competition binding examines the ability of increasing concentrations of unlabeled ligand to compete with [3H]-estradiol for binding to the calf uterine estrogen receptor. The relative binding affinities of the three dihydroxyl ligands, 1-, 2-, and 4-hydroxyestratrien-17β-ol, were 0.22%, 8.74%, and 1.32%, respectively, of that for estradiol (Figure 1; Table I). The unlabeled monohydroxyl ligands had binding affinities of 1.51% for estratrien-17β-ol and 2.48% for 3-hydroxy-estratriene relative to that of estradiol (Figure 1; Table I).

Table II: Cooperativity of [3H]Estradiol Binding to the Calf Uterine Estrogen Receptor[a] in the Presence of Estrogen Analogs

ligand	Hill coeff
[3H]estradiol	1.68 ± 0.05
+equimolar estradiol	1.70 ± 0.08
+1-hydroxyestratrien-17β-ol	1.29 ± 0.04
+2-hydroxyestratrien-17β-ol	1.07 ± 0.10
+4-hydroxyestratrien-17β-ol	1.09 ± 0.04
+estratrien-17β-ol	1.35 ± 0.07
+3-hydroxyestratriene	1.91 ± 0.09

[a] The Hill coefficients for [3H]estradiol binding to the estrogen receptor in the absence and in the presence of unlabeled competitor are the averages ± SEM of 4–6 separate experiments. Receptor concentrations between 2.8 and 5.2 nM were used.

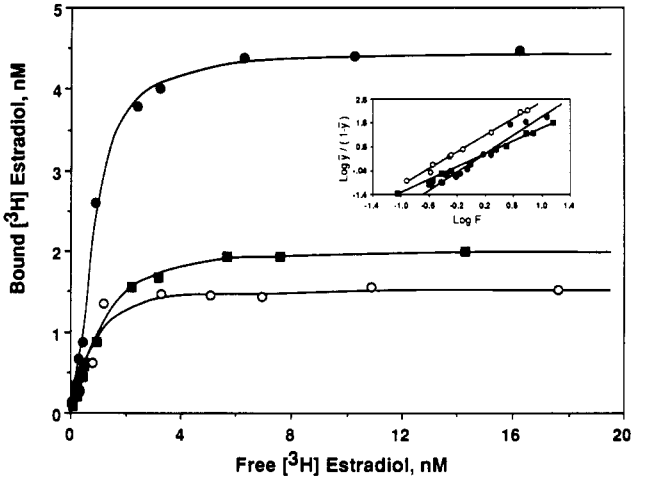

FIGURE 2: Saturation binding analysis of the binding of [3H]estradiol to the estrogen receptor in the presence of a constant molar excess of unlabeled competitor. Saturation binding analysis was performed using ammonium sulfate fractionated calf uterine cytosol as described in Methods. The binding of [3H]estradiol to the estrogen receptor was measured in the absence of competing ligand (●), in the presence of 1-hydroxyestratrien-17β-ol (■), or in the presence of equimolar unlabeled estradiol (○). The corresponding Hill plot is shown as an inset. The Hill coefficients of the data shown are 1.37 ± 0.07 in the presence of 1-hydroxyestratrien-17β-ol; 1.64 ± 0.10 for [3H]estradiol alone; and 1.68 ± 0.09 in the presence of equimolar unlabeled estradiol. These data are representative of four independent experiments.

An analysis of covariance using pooled data for the linear portions of these displacement curves was used to test for parallelism. For the monohydroxyl derivatives, 3-hydroxyestratriene and estratrien-17β-ol, the *P* values were 0.001 and 0.01 and the coefficients of determination were 97.2% and 95.5%, respectively. This indicates that the slopes of the linear portions of the two curves did differ statistically from that of estradiol. For the dihydroxyl derivatives, no significant differences in the slopes were observed, indicating that the binding mechanism of these ligands was similar to that of estradiol.

The calf uterine estrogen receptor binds [3H]estradiol with a maximum Hill coefficient of 1.68 ± 0.05. This observation is consistent with a positive cooperative binding mechanism. The binding mechanism of [3H]estradiol to the estrogen receptor in the presence of the unlabeled ligands was investigated by saturation binding, and the Hill coefficients were determined (Table II; Figures 2–6). Both 1-hydroxyestratrien-17β-ol and estratrien-17β-ol decreased the Hill coefficient to 1.29 ± 0.04 and 1.35 ± 0.07, respectively. In the presence of either 2- or 4-hydroxyestratrien-17β-ol the Hill coefficients were decreased to 1.07 ± 0.10 and 1.09 ± 0.04, respectively. When 3-hydroxyestratriene was added to

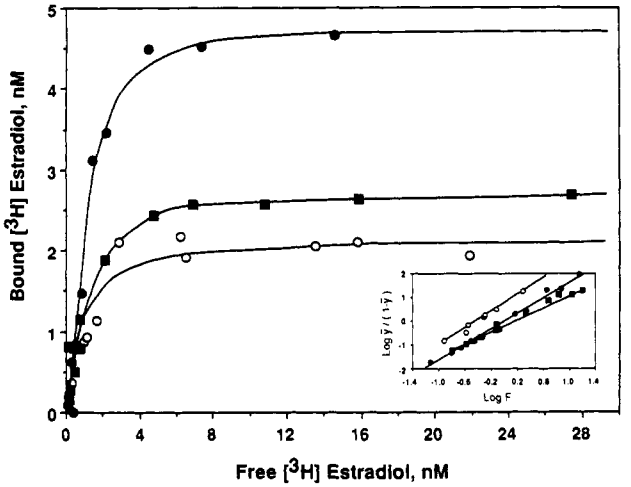

FIGURE 3: Saturation binding analysis of the binding of [^{3}H]estradiol to the estrogen receptor in the presence of estratrien-17β-ol. The binding of [^{3}H]estradiol to the estrogen receptor was measured in the absence of competing ligand (●), in the presence of estratrien-17β-ol (■), or in the presence of equimolar unlabeled estradiol (○). Shown are saturation and Hill (inset) plots of representative experiments. The Hill coefficients of the above data are 1.47 ± 0.12 in the presence of estratrien-17β-ol, 1.64 ± 0.10 for [^{3}H]estradiol alone, and 1.59 ± 0.003 in the presence of equimolar unlabeled estradiol.

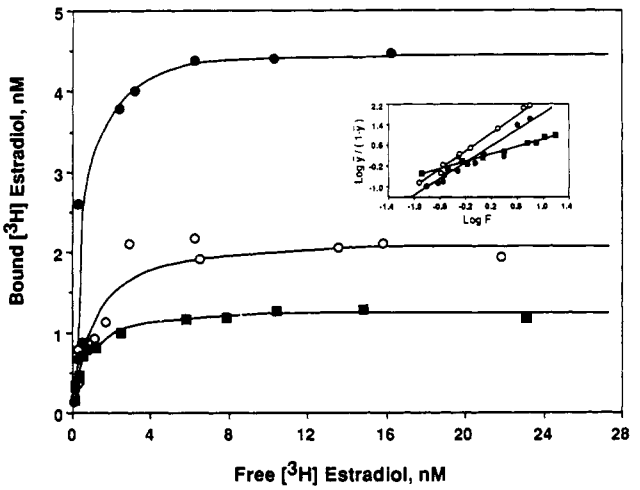

FIGURE 4: Saturation binding analysis of the binding of [^{3}H]estradiol to the estrogen receptor in the presence of 2-hydroxyestratrien-17β-ol. The binding of [^{3}H]estradiol to the estrogen receptor was measured in the absence of competing ligand (●), in the presence of 2-hydroxyestratrien-17β-ol (■), or in the presence of equimolar unlabeled estradiol (○) as described in Methods. Shown are saturation and Hill (inset) plots of representative experiments. The Hill coefficients of the above data are 0.94 ± 0.002 in the presence of 2-hydroxyestratrien-17β-ol, 1.59 ± 0.003 for [^{3}H]estradiol alone, and 1.54 ± 0.13 in the presence of equimolar unlabeled estradiol.

the [^{3}H]estradiol–estrogen receptor mixture, the Hill coefficient increased to 1.91 ± 0.09.

DISCUSSION

Recent studies have demonstrated that different ligands promote different molecular conformations when bound to the estrogen receptor protein (Ruh et al., 1990; Jasper et al., 1985). The proposed consequences of these different ligand–receptor conformations are ultimately seen as changes in biological effect. One basic question is how ligand is able to convey this information to the receptor protein once it does bind. Investigators have proposed that an allosteric site–site interaction on the estrogen receptor dimer is involved (Notides

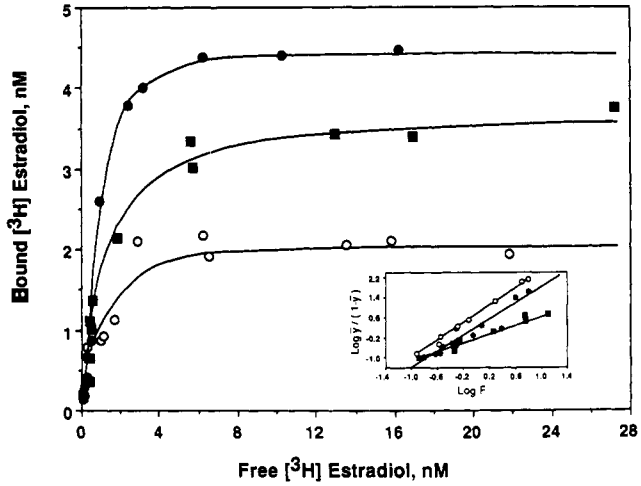

FIGURE 5: Saturation binding analysis of the binding of [^{3}H]estradiol to the estrogen receptor in the presence of 4-hydroxyestratrien-17β-ol. The binding of [^{3}H]estradiol to the estrogen receptor was measured in the absence of competing ligand (●), in the presence of 4-hydroxyestratrien-17β-ol (■), or in the presence of equimolar unlabeled estradiol (○) as described in Methods. Shown are saturation and Hill (inset) plots of representative experiments. The Hill coefficients of the above data are 1.08 ± 0.003 in the presence of 4-hydroxyestratrien-17β-ol, 1.64 ± 0.10 for [^{3}H]estradiol alone, and 1.68 ± 0.09 in the presence of equimolar unlabeled estradiol.

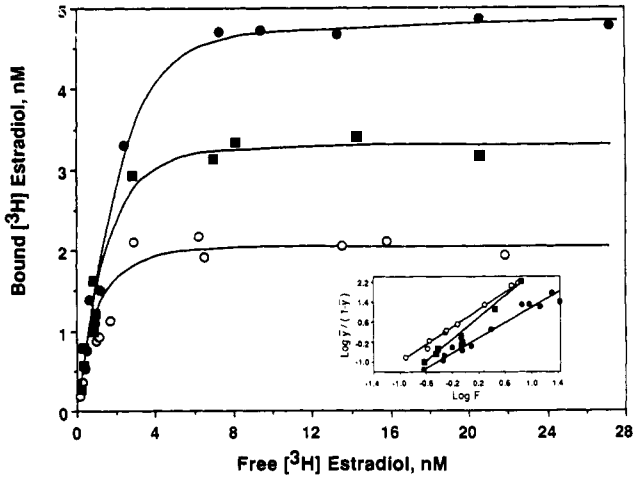

FIGURE 6: Saturation binding analysis of the binding of [^{3}H]estradiol to the estrogen receptor in the presence of 3-hydroxyestratriene. The binding of [^{3}H]estradiol to the estrogen receptor was measured in the absence of competing ligand (●), in the presence of 3-hydroxyestratriene (■), or in the presence of equimolar unlabeled estradiol (○). Shown are the saturation and Hill (inset) plots of representative experiments. The Hill coefficients of the above data are 1.56 ± 0.03 in the presence of 3-hydroxyestratriene, 1.64 ± 0.10 for [^{3}H]estradiol alone; and 1.64 ± 0.08 in the presence of equimolar unlabeled estradiol.

et al., 1981). The conformational properties of the estrogen receptor have been shown by using a variety of techniques to differ when bound to ligands such as 4-hydroxytamoxifen (Sabbah et al., 1991; Pavlik et al., 1985; Ruh et al., 1990; Fritsch et al., 1992), H1285 (Kleene et al., 1984; Ruh et al., 1990), ICI 164,384 (Fawell et al., 1990), or clomiphene (Sasson & Notides, 1982) versus estradiol. In no instance, however, has the binding mechanism, which is indicative of conformational changes, been systematically studied for a set of structurally altered estradiol analogs.

Cooperativity is usually prominent at switch points in biological systems, allowing the activity of a protein to be controlled by low critical ligand concentrations. Furthermore, the positive cooperative equilibrium binding of estradiol to the estrogen receptor is characteristic of the receptor's

activation process (Sasson & Notides, 1983). Given the functional significance of the positive cooperative [^{3}H]-estradiol–estrogen receptor interaction, we sought to characterize it further. In this study five different estradiol analogs, including mono- and dihydroxyl ligands, were tested. In the monohydroxyl analogs, either the A- or the D-ring hydroxyl had been removed. In the dihydroxyl analogs, the phenolic hydroxyl group had been moved around the A-ring. Using competition and saturation binding assay, we were able to distinguish between those structural features on the steroid molecule required for binding site competition from those critical to the positive cooperative binding mechanism.

Competition binding assay showed that any change in the structure of the steroid molecule resulted in a decreased affinity for the estrogen receptor relative to that of estradiol. In our experiments, estratrien-17β-ol had 1.5% of the binding affinity of estradiol, which agrees well with the results of Chernayaev et al. (1978). However, we found 3-hydroxyestratriene had 2.5% of the binding affinity of estradiol, whereas Chernayaev et al. (1978) reported a value of 14%. Two major differences could account for these observations. First, we used bovine, rather than rabbit, uterus; there could be differences between estrogen receptors from these two species. Second, our experiments were carried out at 25 rather than 4 °C. Sasson and Notides (1983) have shown that estrone and estriol have different binding mechanisms with the estrogen receptor at different temperatures: at 0 °C, their binding to the receptor is positive cooperative, whereas at 30 °C their binding is noncooperative. We have used 25 °C for our experiments, rather than lower temperatures, because it is closer to the physiological temperature. We also examined the slopes of their displacement curves to compare their binding mechanism for the receptor with that of estradiol. For all three dihydroxyl analogs, the slopes were similar to that of estradiol. The slopes of the displacement curves for the monohydroxyl analogs, however, were significantly different from that of estradiol, indicating that the binding mechanism of these ligands was different from that of estradiol.

The effect that changing the hydroxyl group position or that removing it had on the positive cooperativity of [^{3}H]-estradiol binding to the estrogen receptor was then examined by saturation binding assay. When the phenolic (A-ring) hydroxyl group was moved to the 1 position (1-hydroxy-estratrien-17β-ol) or omitted altogether (estratrien-17β-ol), the positive cooperativity was reduced from 1.68 to 1.29 and 1.35, respectively. The presence of ligands whose A-ring hydroxyl group had been moved to either the 2 or the 4 position (2- or 4-hydroxyestratrien-17β-ol) nearly abolished the positive cooperativity; the Hill coefficient was decreased to 1.07 and 1.09, respectively. Finally, when the A-ring hydroxyl group was left intact at the 3 position but the D-ring hydroxyl group was removed (3-hydroxyestratriene), the positive cooperativity increased to 1.91. As expected, the positive cooperativity of [^{3}H]estradiol binding by the estrogen receptor was unaffected by the presence of unlabeled equimolar estradiol. This showed that the observed changes in the positive cooperative [^{3}H]-estradiol–estrogen receptor interaction in the presence of structurally altered ligands were not due to a decrease in the specific activity of the labeled ligand. Interestingly, the binding of [^{3}H]estratrien-17β-ol to the estrogen receptor in the absence of estradiol was noncooperative; $n_H = 0.85 \pm 0.03$ (data not shown). Estradiol by itself bound to the receptor with a Hill coefficient of 1.7. In the presence of estratrien-17β-ol, cooperativity of [^{3}H]estradiol binding was reduced but not eliminated, as shown by the Hill coefficient of 1.35. These results suggest that not only does estratrien-17β-ol bind noncooperatively to the receptor but it also blocks conformational changes required for positive cooperativity.

These observations suggest that moving the phenolic hydroxyl group to any position on the A-ring other than the 3-position, or removing it altogether, but leaving the D-ring hydroxyl group in place diminished the positive cooperativity. These results show that the phenolic hydroxyl at C3 was important not only for affinity but for cooperativity as well. The studies presented here also indicate that the 17β-OH can be removed (3-hydroxyestratriene) without blocking ligand-induced conformational changes. This contrasts with a previous model which had suggested that the D-ring hydroxyl was important for long-range conformational changes (Duax et al., 1981).

The observed reduction in the Hill coefficient in the presence of all but one of the ligands tested in consistent with either less efficient dimerization of the estrogen receptor or reduced site–site interaction within the receptor dimer. The data presented here do not allow absolute distinctions between these possibilities to be made. At the concentrations of estrogen receptor used in these experiments, 2.8–5.2 nM, the estrogen receptor behaves as a dimer (Notides et al., 1981). Since the ligands used in these experiments lack the bulky side chains which have been suggested to affect dimerization, it is more likely that site–site interaction was reduced. Conversely, the presence of 3-hydroxyestratriene, which increased the Hill coefficient of [^{3}H]estradiol binding, could promote either increased dimerization or greater site–site interaction within the receptor dimer. Since the calf uterine estrogen receptor is probably dimeric at the concentrations used, we favor the idea that site–site interactions were enhanced.

Recently, other investigators have studied the activity of these same A- and D-ring analogs in MCF-7 cells. All five estradiol analogs bring about tight nuclear binding and processing of the estrogen receptor and are able to activate estrogen-response-element-regulated CAT expression (Pilat et al., 1993; VanderKuur et al., 1993). Furthermore, progesterone receptor induction, a characteristic response of the estradiol–estrogen receptor interaction, was strongly stimulated by all of these ligands except 2- and 4-hydroxyestratrien-17β-ol (VanderKuur et al., 1993). Our findings that the presence of either of these dihydroxyl ligands induces a switch from a positive cooperative to a noncooperative binding mechanism may help to interpret these results. The impaired function of the 2- and 4-hydroxyestratrien-17β-ol ligands in progesterone receptor induction is consistent with the reduced cooperativity shown in our studies.

Cathepsin D (Westleg & Rochefort, 1980; Morisset et al., 1986) and pS2 (Masiakowski et al., 1982) are also estrogen-responsive genes in MCF-7 cells. The estradiol analogs 3-hydroxyestratriene, estratrien-17β-ol, and 1-hydroxy-estratrien-17β-ol were capable of inducing synthesis of both the pS2 and the cathepsin D mRNA in MCF-7 cells (Pilat et al., 1993). While 2-hydroxyestratrien-17β-ol ligand was able to induce the pS2 gene, it was only marginally effective in the induction of the cathepsin D gene (Pilat et al., 1993). The 4-hydroxyestratrien-17β-ol ligand was less able to induce either gene (Pilat et al., 1993).

Both 2- and 4-hydroxyestratrien-17β-ol reduced the Hill coefficient of estradiol binding to approximately 1.1, yet they have markedly different effects on induction of gene expression. Their effects on gene expression may therefore reflect specific requirements of individual genes for optimal induction, such as different combinations of regulatory transcription factors.

The results also imply that the conformations of these ligand–receptor complexes are different from each other as well as from estradiol.

It has been speculated that the binding of structurally altered ligands to the estrogen receptor could affect gene transcription by interfering with dimerization of the receptor complex (Lannigan & Notides, 1989; Fawell & White, 1992), by influencing the hormone binding domain transactivation function, TAF-2 (Tora et al., 1989; Ing et al., 1992), by changes in the binding of the estrogen receptor complex to the estrogen-response element (Carson-Jurica et al., 1990), or by unique ligand-induced receptor conformations which could interfere with protein–protein interactions in the vicinity of the TAF-2 site on the estrogen receptor complex (VanderKuur et al., 1993). Our studies do not directly address dimerization or binding of the ligand–receptor complex to an estrogen-response element. Comparing changes in the Hill coefficients does allow us to compare the effects that these ligands have on the changes in structure which allow communication between binding sites. What we found was that, with the exception of 3-hydroxyestratriene, all ligands used here were somehow able to interfere with the site–site estradiol binding interaction. Our findings are consistent with the view that different ligand–receptor complexes have different molecular conformations and thereby provide a mechanism for explaining the differential effects of these complexes on gene induction. For example, ligand-induced alterations in the receptor could promote, limit, or hinder the accessibility of regions critical to protein–protein interactions.

In allosteric proteins the binding of one molcule of ligand induces structural changes in the protein which result in altered affinities for the binding of subsequent molecules of ligand. Two models which have been proposed to explain the cooperative behavior of allosteric proteins are the sequential interaction, or induced-fit, model (Koshland et al., 1966) and the concerted transition, or symmetry, model (Monod et al., 1965). The induced-fit model assumes that sequential changes in protein conformation result in altered affinities for ligand at vacant sites, the transitions to which are progressive and cumulative. The concerted model assumes that a preexisting equilibrium exists between high- and low-affinity conformational states; ligand binding displaces the equilibrium toward one state or the other. In this model, the transitions are all or none. Finally, other models propose that combinations of concerted and sequential processes exist (Perutz, 1970; Lee & Karplus, 1983; Johnson et al., 1984). Neither the model which best applies nor the structural framework which modulates the allosteric transitions for the estrogen receptor has been determined.

One of the basic assumptions of both the concerted and the induced-fit models is that allosteric proteins are oligomeric, with identical binding sites (Monod et al., 1965; Koshland et al., 1966). This is in keeping with the observations that high-affinity estradiol and DNA binding requires a receptor dimer (Weichman & Notides, 1977; Skafar & Notides, 1985, 1987; Skafar, 1991). However, the two-state concerted model cannot account for either the different binding mechanism of 4-hydroxytamoxifen for the estrogen receptor or tamoxifen's mixed agonist and antagonist activity. This is best reconciled with an induced-fit model. The suggestion that 4-hydroxytamoxifen induces a distinct conformation in the receptor which may hinder access to transcription factors (Klinge et al., 1992) is also consistent with the induced-fit model. The effects of the ligands described in this paper on the Hill coefficient and on gene induction correspond best with the induced-fit model. Finally, the presence of amino-substituted estradiol ligands appears to actually produce a negatively cooperative binding mechanism (Schwartz and Skafar, manuscript in preparation); these findings cannot be explained by a concerted model. We therefore suggest that although the estrogen receptor displays some of the characteristics of the concerted model, its effects on the induction of different genes and the modulatory effects of different ligands on the receptor's positive cooperative binding mechanism are best described using the induced-fit model.

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