Estrogen Receptor Transformation to a High-Affinity State without Subunit–Subunit Interactions†

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ABSTRACT: We have tested the ability of monomeric rat estrogen receptor (generated by dissociating the native receptor aggregate with KCl and binding to hydroxylapatite) to undergo heat-induced transformation to a state with altered chromatographic properties and estradiol dissociation kinetics. Participation of nonadsorbed cytosolic components in the transformation process was eliminated by washing the adsorbed receptor prior to incubation at 28–30 °C for 30 min. We found that monomeric estradiol–receptor complexes can be converted to a form which coelutes with transformed receptor during hydroxylapatite chromatography and which has a slow estradiol dissociation rate characteristic of transformed receptor. Monomeric receptor does not, however, display cooperative ligand-binding behavior, strongly suggesting adsorbed monomers cannot dimerize or interact with other adsorbed cytosol proteins. Maintenance of the monomer form was further verified by the sedimentation of eluted receptor solely as a 4S species. Although conversion of monomers to the slow-dissociating state was incomplete (≃80%) on hydroxylapatite, eluted receptor showed only a slow dissociation rate component. The data are consistent with a model in which receptor monomers are in equilibrium between low-affinity and high-affinity conformations. Dimerization of the high-affinity receptor may shift the equilibrium to greatly favor the high-affinity (transformed) state.

The estrogen receptor protein is known to exist in two states. One form predominates in animals treated with estrogenic compounds and can be isolated from the nuclear fraction of target tissues. A cytosol form predominates in the absence of steroid (Jensen et al., 1968; Shyamala & Gorski, 1969). In vitro, the cytosolic receptor can be transformed to the nuclear-derived form by treatment with estrogens for prolonged periods at low temperature or for short periods at elevated temperature or elevated ionic strength. The transformed or nuclear-derived estrogen receptor differs from the nontransformed or cytosolic receptor by several criteria, including an increased affinity for estradiol (Weichman & Notides, 1977), DNA (Yamamoto, 1974), and nuclei (Brecher et al., 1967; Jensen et al., 1972; Gschwendt & Hamilton, 1972), altered chromatographic behavior (Molinari et al., 1977; Miller & Toft, 1978; Notides et al., 1981), and, in the immature rat and calf, appearance as a dimer with a sedimentation coefficient of 5 S (Jensen et al., 1971; Gschwendt & Hamilton, 1972). The nontransformed receptor sediments as a 4S monomer in high-salt buffers and as an 8S aggregate in low-salt buffers.

The combined effects of enhanced steroid affinity and oligomer formation manifest themselves with steroid-binding kinetics that show evidence of positive cooperativity at sufficiently high receptor concentrations (Notides et al., 1981; Sasson & Notides, 1983). Such behavior has been interpreted to suggest that site–site interactions within the receptor dimer are responsible for the increased affinity for steroid characteristic of the transformed state. It also has been suggested that the transformation event is mediated by other cytosolic proteins, either by enzymatic methods (Puca et al., 1977) or by formation of a complex with the receptor (Thrower et al., 1976; Thampan & Clark, 1981).

In order to test the presumptive role of subunit–subunit interactions and nonreceptor proteins in transformation, we have dissociated the estrogen receptor into monomers by treatment with high-salt buffers and bound these monomers to hydroxylapatite. Monomers do not exhibit cooperative ligand-binding properties yet, when heated, attain steroid dissociation kinetics and hydroxylapatite chromatography elution characteristics similar to transformed receptor. These and other observations are consistent with a mechanism in which estrogen induces a conformational change of the monomeric receptor and dimerization occurs as a subsequent and separate event.

Experimental Procedures

Materials. 17β-[2,4,6,7-^{3}H]Estradiol (90 and 104 Ci/mmol) was purchased from New England Nuclear and Amersham and was 90–98% pure by thin-layer chromatography (TLC)[1] (1:1 benzene:ethyl acetate). Immature female rats were obtained from Holtzman (Madison, WI) and used at the ages of 20–22 days. Hydroxylapatite (HAP), Bio-Gel HT, was from Bio-Rad Laboratories and prepared for use as described (Williams & Gorski, 1974). Other chemicals were reagent grade.

Equilibrium Binding. All procedures were performed at 0–4 °C except where indicated. Uteri were dissected and immediately placed in ice-cold buffer containing 10 mM Tris-HCl, pH 7.4 (25 °C), 1.5 mM EDTA, 0.5 mM dithiothreitol, and 0.02% (w/v) sodium azide. The uteri were rinsed several times with buffer to remove serum contaminants and homogenized at about three uteri per milliliter of buffer in a glass–glass tissue grinder chilled in an ice bath. Cytosol was prepared by centrifugation at 130000g_{av} for 1 h. Aliquots containing approximately 0.25 pmol of receptor were placed in glass test tubes. [^{3}H]Estradiol in 50 mM Tris-HCl, pH 7.4 (25 °C), 0.5 mM dithiothreitol, and 0.02% (w/v) sodium azide (TD) buffer was added to give final concentrations ranging

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[1] Abbreviations: HAP, hydroxylapatite; DES, diethylstilbestrol; TD, 50 mM Tris-HCl, pH 7.4 (25 °C), 0.5 mM dithiothreitol, and 0.02% (w/v) sodium azide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid.

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between 0.05 and 20 nM. All incubation mixtures contained 1% (v/v) ethanol. Some also contained a 200-fold excess of diethylstilbestrol (DES) over the [^{3}H]estradiol concentration for determination of nonspecific binding. Following incubation at 0 °C for 18 h, 0.25 mL of a 70% (packed volume/suspended volume) slurry of HAP was added to each tube. After being mixed for 30 min, the samples were centrifuged at 1000g for 1 min and aliquots removed for estimation of free steroid concentrations. The hydroxylapatite pellets were rapidly washed 3 times each by resuspension in 3 mL of TD buffer and centrifugation at 1000g for 1 min. The final pellet was extracted with 1 mL of ethanol. Aliquots of 0.5 mL were counted in 3.5 mL of 10% (v/v) Bio-Solv 3 (Beckman), 0.5% (w/v) 2,5-diphenyloxazole, 0.03% (w/v) 1,4-bis[2-(5-phenyloxazolyl)]benzene, and toluene-based scintillation cocktail at 32% efficiency. Aqueous samples (10–50 μL) were counted in the same cocktail at 42% efficiency. Specific binding was calculated as the [^{3}H]estradiol bound in the absence of DES competitor minus the [^{3}H]estradiol bound in the presence of DES competitor in an incubation mixture of equivalent free [^{3}H]estradiol concentration. Thus, at a given concentration of total [^{3}H]estradiol (Blondeau & Robel, 1975)

$$B_{sp} = B_{tot} - (F_{tot}/F_{nonsp})B_{nonsp}$$

where B_{sp}, B_{tot}, and B_{nonsp} are the concentrations of specifically bound [^{3}H]estradiol, [^{3}H]estradiol bound in the absence of DES competitor, and that bound in its presence. F_{tot} and F_{nonsp} are the corresponding free [^{3}H]estradiol concentrations. Data are presented in the format of Scatchard plots (Scatchard, 1949). Specific binding between 0.1 and 0.9 fractional saturation of receptor also is shown in Hill plots (Hill, 1910).

For binding studies with HAP-adsorbed receptor, HAP was added to each cytosol aliquot. In some cases, the cytosol and HAP were equilibrated with 0.4 M KCl for 0.5 h prior to mixing. Following 0.5 h of mixing, the samples were washed with TD buffer or TD buffer plus 0.4 M KCl as described above and resuspended in TD buffer containing hormone. Following incubation at 0 °C for 18 h with occasional gentle mixing, the samples were centrifuged and aliquots removed for measurement of free steroid as described above. Bound steroid was measured also as described above.

Aliquots of unlabeled cytosol were reserved for determination of receptor stability. Single saturating doses of [^{3}H]estradiol (10 nM) plus or minus 2 μM DES competitor were added to aliquots at the beginning and end of each incubation and mixed at 0–4 °C for 1 h. If the receptor was not already HAP bound, HAP was added, and the samples were processed as described above.

Isokinetic Sedimentation. Convex exponential 5–16.5% (w/w) sucrose gradients in TD buffer plus 0.4 M KCl and 1.5 mM EDTA were prepared in polyallomer tubes as per Noll (1967) by using the specifications of McCarty et al. (1974) for an SW 56 rotor (Beckman) and particles of density 1.33 g/cm^3 at 4 °C. All samples included ^{14}C-labeled ovalbumin (3.6 S) as an internal size standard. Centrifugation was for 16 h at 50000 rpm at 4 °C.

Hydroxylapatite Chromatography. Cytosol prepared as described above from 10 uteri was equilibrated with 10 nM [^{3}H]estradiol at 0 °C for 1 h. For some samples, transformed receptor was generated by heating at 30 °C for 0.5 h. HAP (3 mL) was added and the slurry mixed on ice an additional 0.5 h. After centrifugation at 1000g for 1 min, the HAP pellet was washed 3 times with 3 mL of TD buffer plus 0.4 M KCl. At this time, some samples were reequilibrated with 10 nM [^{3}H]estradiol, heated at 30 °C for 0.5 h, and rewashed. Slurries were then poured into 5-mL plastic syringes plugged with silanized glass wool. The columns (bed volume of 2 mL) were connected to a reservoir via a peristaltic pump and washed with 50 mL of TD buffer plus 0.2 M KCl at a flow rate of about 7 mL/h. Essentially all the unbound steroid was removed during these washes. [^{3}H]Estradiol–receptor complexes were then eluted with a linear gradient of 0–0.15 M phosphate. Fractions (2 mL) were collected and analyzed for protein by the absorbance at 280 nm and for phosphate by conductivity. Aliquots (0.1 mL) were measured for radioactivity by scintillation counting in 4 mL of scintillation cocktail (see above) and 0.1 mL of water at 43% efficiency.

Dissociation Kinetics. Uterine cytosol prepared as described above was equilibrated with 5 nM [^{3}H]estradiol plus or minus 1 μM DES competitor in TD buffer at 0 °C for 1 h. Some samples were then incubated at 28 °C for 0.5 h. After the samples were cooled, aliquots (0.1 mL) containing approximately 0.25 pmol of receptor were placed in glass test tubes, and 0.1 mL of TD buffer containing 2 μM DES was added. [^{3}H]Estradiol dissocation was initiated by transferring tubes to a 28 °C water bath. Dissociation was stopped after 1–80 min of incubation by transferring tubes to an ice bath and adding 2 mL of ice-cold TD buffer. HAP slurry (0.25 mL) was added, and the samples were processed for determination of [^{3}H]estradiol–receptor complexes as described above. Parallel incubations were performed with samples not receiving excess DES to measure receptor stability. Data are plotted as the log of the fraction of labeled complexes remaining after correction of each time point, individually, for nonspecific binding (<10%) and receptor inactivation (<5%). Dissociation rate constants and the fractional contribution of each component of biphasic curves were determined by extrapolation (Ray & Koshland, 1961). Kinetic constants are presented as mean ± SE (number of determinations).

Dissociation studies with HAP-adsorbed receptor were done similarly except HAP was added to each assay tube prior to the dissociation assay. The HAP mixture was washed twice by mixing with 3 mL of TD buffer and centrifuging, resuspended in 1 volume of TD buffer containing 5 nM [^{3}H]estradiol plus or minus 1 μM DES, and either kept at 0 °C or heated at 28 °C for 0.5 h. The samples were washed twice more before being resuspended in 1 volume of TD buffer containing 2 μM DES. Dissociation of [^{3}H]estradiol was monitored by isotopic dilution as described above.

Monomeric estrogen receptors formed by treatment of cytosol with 0.4 M KCl at 0 °C for 0.5–1 h were adsorbed onto HAP before addition of [^{3}H]estradiol. This prevented salt-induced transformation. The HAP mixture was washed once with TD buffer plus 0.4 M KCl and twice with TD buffer. [^{3}H]Estradiol (5 nM) plus or minus 1 μM DES was added in TD buffer, and slurries were gently mixed at 0 °C for 1 h. Some samples were then heated at 28 °C for 0.5–1.5 h. Most of the unbound steroid was removed by two washes of the HAP mixture with TD buffer. The samples were then resuspended in 1 volume of TD buffer plus 2 μM DES and processed further as described above.

Results

Adsorption of the Estrogen Receptor onto Hydroxylapatite. For clear interpretation of our results, it is imperative that receptor be tightly bound to HAP even at elevated temperature and ionic strength. If receptor deadsorbed from the HAP during our experiments, one would not be able to rule out involvement of protein–protein interactions in the transformation process.

We tested the efficiency of adsorption and retention of receptor by HAP by incubating various amounts of labeled

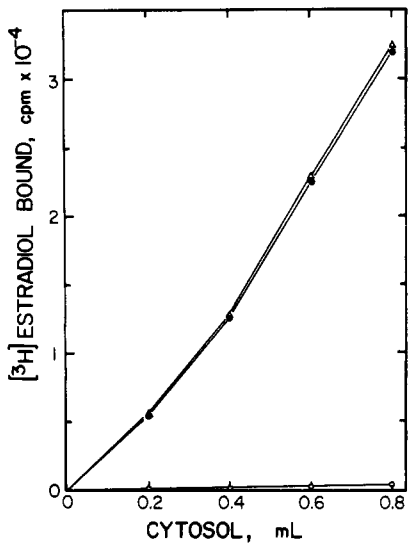

FIGURE 1: Efficiency of adsorption and retention of estrogen receptors by hydroxylapatite. Cytosol was prepared from a homogenate of eight uteri in 8 mL of TD buffer and equilibrated with 10 nM [^{3}H]estradiol with or without 2 μM DES at 0 °C for 1.5 h. Duplicate aliquots (0.2–0.8 mL) were added to 0.2 mL of HAP suspension and 2 mL of TD buffer and mixed at 0 °C for 0.5 h. The samples were centrifuged at 4 °C at 1000g for 2 min and the supernatants decanted into a separate set of tubes. The HAP pellets were resuspended in 2 mL of TD buffer and incubated at 30 °C for 0.5 h. The samples were centrifuged at room temperature as above and the supernatants decanted into a third set of tubes. The supernatant samples were mixed with 0.2 mL of HAP and all samples washed 3 times with TD buffer. Specifically bound cpm was determined as described under Experimental Procedures. (△) Total [^{3}H]estradiol–receptor complexes. (●) HAP-bound complexes. (○) Sum of supernatant complexes.

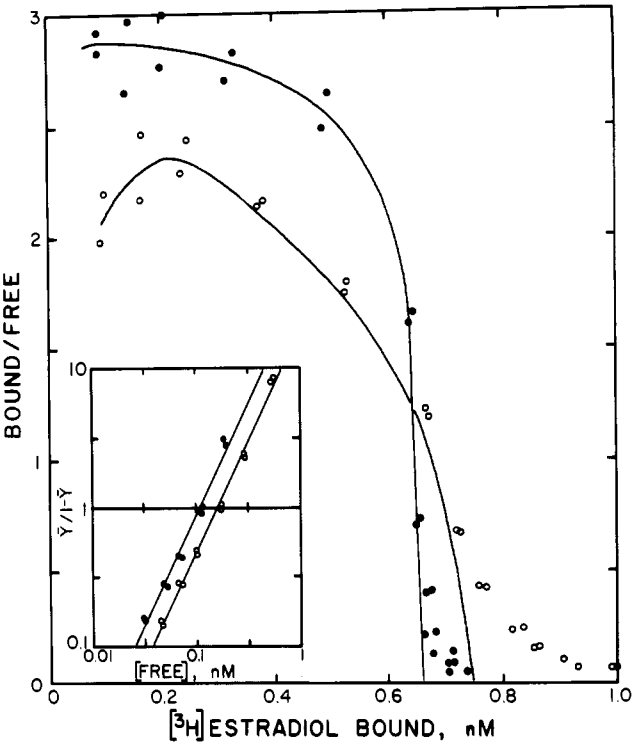

FIGURE 2: Scatchard plot of equilibrium binding at 0 °C of [^{3}H]-estradiol to estrogen receptors in solution or adsorbed to hydroxylapatite. Lines represent receptor binding after correction of the data points for the contribution of type II sites (see Results). Inset: Hill plots of corrected data. Lines represent linear least-squares fit to the data. (●) Binding to receptor in solution; receptor concentration = 0.66 nM, $S_{0.5}$ = 0.11 nM, n_H = 1.60. (○) Binding to receptor adsorbed to HAP; receptor concentration = 0.75 nM, $S_{0.5}$ = 0.18 nM, n_H = 1.59.

cytosol with a constant quantity of HAP at 0 °C. After one wash with TD buffer at 0 °C, a second wash was performed at 30 °C. The combined [^{3}H]estradiol–receptor complexes in the two washes amounted to less than 1% of the fraction remaining bound to the HAP (Figure 1). Identical results were obtained by using buffers containing 0.15 and 0.4 M KCl. Thus, HAP appears to be a satisfactory solid support for our studies. Quantitative adsorption and retention of receptor by HAP at elevated temperatures had been suggested but not convincingly demonstrated by previous studies (Garola & McGuire, 1977; de Boer & Notides, 1981a).

Equilibrium Binding of Estradiol to Receptor Adsorbed to Hydroxylapatite. In order to verify that adsorption of the estrogen receptor to HAP does not alter its interaction with steroid, we compared saturation analyses of the adsorbed receptor and the receptor free in solution. Similar results were obtained for the receptor in TD buffer at 0 °C with or without preadsorption to HAP. Scatchard plots of the data suggest the presence of both positive cooperativity (convex-upward curvature) and binding-site heterogeneity (convex-downward curvature) (Figure 2). We occasionally observe nonlinearity of Scatchard plots at high levels of receptor saturation and attribute the phenomenon to type II binding sites which have been previously characterized (Clark et al., 1978). These sites are apparently unrelated to the classical estrogen receptor of interest in this study, and therefore, we have corrected for their contribution to the binding isotherms by the method of Rosenthal (1967) assuming a dissociation constant of 30 nM for type II sites (Clark et al., 1978). The corrected data, replotted in Hill plots of Figure 2 (inset), reveal similar Hill coefficients, n_H = 1.6, for both cases. Also, ligand concentrations at which the receptor populations are half-maximally saturated are similar, $S_{0.5}$ = 0.17 and 0.13 nM with and without HAP preadsorption, respectively. Thus, HAP adsorption and removal of soluble cytosol components neither affect the binding capacity or affinity of the receptor for estradiol [see also Garola & McGuire (1977)] nor disrupt subunit interactions involved in cooperative ligand-binding behavior.

We have consistently observed convex-upward Scatchard plots at receptor concentrations between 0.5 and 2 nM with or without HAP preadsorption [n_H = 1.51 ± 0.02 (10)]. With receptor concentrations below 0.5 nM, only linear Scatchard plots were observed. An elevated receptor concentration may be necessary to facilitate receptor oligomerization.

It is unlikely that the apparent cooperativity is artifactual due to nonequilibrium conditions, receptor degradation, or systematic errors in measurement of the free and bound ligands. Apparent binding equilibrium was attained by 6 h of incubation and was stable to at least 20 h. Though 10% losses in steroid-binding activity were typical, receptor inactivation should only result in a change in the slope, not the convexity, of Scatchard plots (Yeakley et al., 1980). We have eliminated the error associated with estimating the free ligand concentration by the difference between total and bound ligand concentrations by measuring the free concentration directly. The free values we obtain match closely those calculated indirectly from the total and bound concentrations by using the correction method of Blondeau & Robel (1975). Nonspecific binding was generally less than 10% of total binding and was a linear function of free steroid concentration. We conclude that our results are most likely due to authentic binding-site interactions within receptor aggregates.

This is supported by the complete elimination of cooperativity by first dissociating the receptor into 4S monomers by treatment with 0.4 M KCl and adsorbing the monomers to HAP. The data graphed on Scatchard plots (Figure 3) were

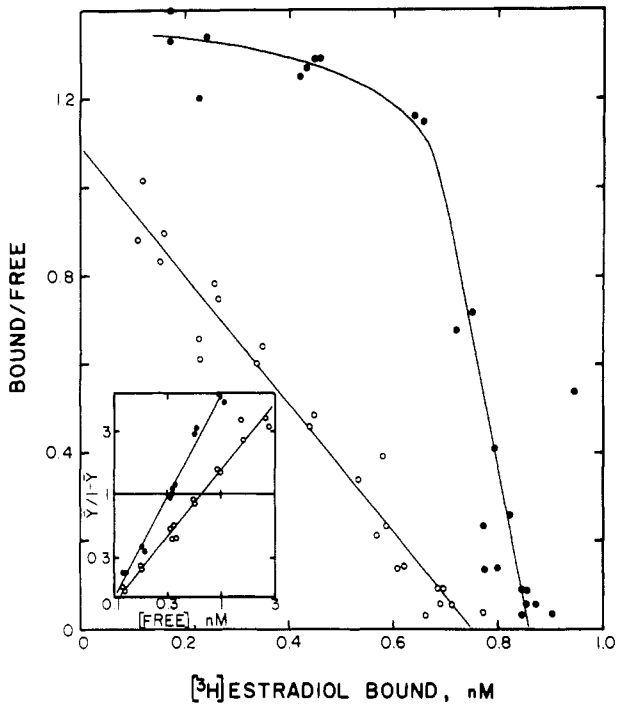

FIGURE 3: Scatchard plot of equilibrium binding at 0 °C of [^{3}H]-estradiol to native and monomeric estrogen receptors. Inset: Hill plots. (●) Binding to receptor in solution; receptor concentration = 0.85 nM, $S_{0.5}$ = 0.32 nM, n_H = 1.54. (○) Binding to monomeric receptor generated by treatment with 0.4 M KCl and adsorption onto HAP; receptor concentration = 0.73 nM, $S_{0.5}$ = 0.64 nM, n_H = 1.01.

linear and on Hill plots (Figure 3, inset) gave slopes of 1.01 ± 0.01 (3). The dissociation constant for the monomeric receptor in TD buffer at 0 °C is $K_{d,monomer} = S_{0.5} = 0.69 \pm 0.09$ nM (3). Loss of cooperativity is not due to a nonspecific salt effect. With KCl treatment alone, conversion to the 5S dimer occurs unimpeded, and cooperativity is unaffected. Identical saturation curves are obtained for receptor in solution with or without 0.4 M KCl addition (receptor concentration = 1.0 nM, $S_{0.5}$ = 0.7 nM, n_H = 1.5).

That receptors treated in the above manner were maintained as monomers was supported by results of sucrose gradient sedimentation of complexes eluted from HAP with 0.3 M phosphate. Only complexes with sedimentation coefficients of about 4 S [4.37 ± 0.04 S (8)] in 0.4 M KCl were observed whether or not the complexes were heated at 30 °C for 0.5 h while bound to HAP to promote transformation. Estradiol–receptor complexes which were heated at 30 °C for 0.5 h prior to adsorption onto and elution from HAP, however, were found to sediment as a 3:1 mixture of 4S and 5S species [4.46 ± 0.10, 5.74 ± 0.04 S (2)] rather than solely 5 S. This indicates high phosphate concentrations may partially dissociate dimeric receptor to monomers.

These results demonstrate that estrogen receptor monomers are incapable of interacting while bound to HAP. Furthermore, they suggest it is unlikely that other cytosol proteins bound to HAP would be capable of interacting with HAP-adsorbed receptor as well.

Hydroxylapatite Chromatography. Nontransformed and transformed estrogen receptors were distinguished by chromatography on HAP. Unheated or nontransformed receptor eluted as a narrow peak at 0.07 M phosphate, and heat-transformed receptor eluted as a broad peak centered around 0.04 M phosphate in buffer containing 0.2 M KCl (Figure 4). The bulk of the 280-nm-absorbing material eluted at 0.01–0.05 M phosphate. Nonspecific binding was low and uniformly distributed throughout the eluate.

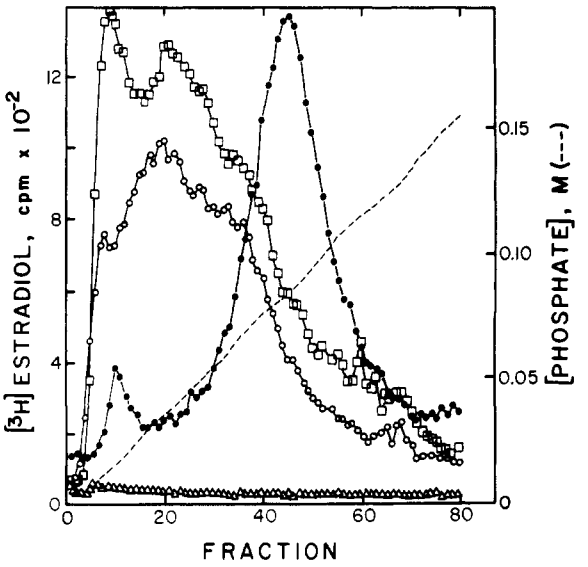

FIGURE 4: Hydroxylapatite chromatography of transformed and nontransformed estrogen receptors. [^{3}H]Estradiol–receptor complexes were equilibrated at 0 (●, □) or 30 °C (○) for 0.5 h and chromatographed on 2-mL HAP columns in TD buffer plus 0.2 M KCl (see Experimental Procedures). (□) Complexes heated at 30 °C for 0.5 h after adsorption to HAP and washing with 0.4 M KCl. (△) Elution profile of cytosol equilibrated with a 200-fold excess of DES in addition to [^{3}H]estradiol.

If, however, unheated receptor was bound to HAP, washed with 0.4 M KCl buffer, and then incubated as one would to transform receptor in solution (30 °C for 0.5 h in the presence of [^{3}H]estradiol), the receptor eluted with a profile similar to a transformed species (Figure 4). Similar results were obtained in nine experiments. Transformation was estrogen dependent and temperature dependent although prolonged exposure to high-salt buffers at 4 °C induced partial conversion to the early-eluting form.

These results demonstrate that the change in the estrogen receptor elution profile does not require involvement of cytosol components which were removed during the wash steps. Since the extensive KCl treatment should have dissociated the receptor into monomers, intersubunit interactions also may not be required. As we had difficulty obtaining complete separation of nontransformed from transformed receptor during HAP chromatography, we decided to use a more quantitative indicator of receptor transformation, steroid dissociation kinetics (Weichman & Notides, 1977), to further investigate the ability of monomers to acquire characteristics of the transformed state.

Dissociation Kinetics of Estradiol–Receptor Complexes Adsorbed to Hydroxylapatite. The dissociation of [^{3}H]estradiol from the unheated rat estrogen receptor in TD buffer is biphasic with rate constants of $k_f = (3.8 \pm 0.0) \times 10^{-3}$ s^{-1} (3) for the fast component and $k_s = (3.3 \pm 0.2) \times 10^{-4}$ s^{-1} (2) for the slow component (Figure 5A,B). The fast-dissociating component comprised 29% of the dissociable binding. Only the slow component [$k_s = (3.3 \pm 0.3) \times 10^{-4}$ s^{-1} (3)] was observed following heating at 28 °C for 40 min, indicative of transformation to the higher affinity state (Figure 5A). The ratio of the dissociation rate constants of the high-affinity and low-affinity states is $k_s/k_f = 0.087$ at 28 °C. This value is 2.6-fold larger than that measured for the calf estrogen receptor (0.033; Weichman & Notides, 1977) and indicates the conformational transition associated with the change in affinity is greater for the calf protein than for the rat protein.

When [^{3}H]estradiol–receptor complexes are first adsorbed onto HAP in TD buffer and then dissociation is monitored,

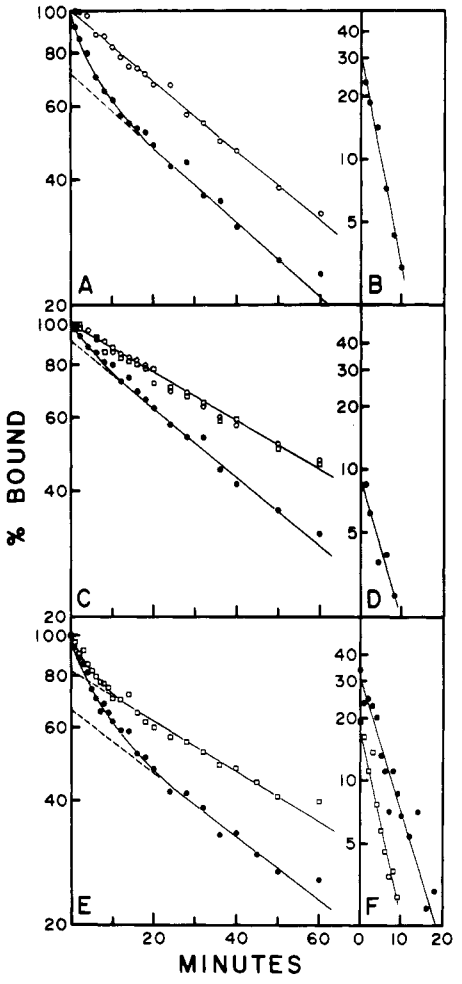

FIGURE 5: Dissociation of [^{3}H]estradiol–receptor complexes in solution or adsorbed to hydroxylapatite. (A) Receptor in solution. [^{3}H]Estradiol–receptor complexes were equilibrated at 0 (●) or 28 °C (○) for 0.5 h, and dissociation of the complexes at 28 °C was monitored by isotopic dilution with a large excess of DES (see Experimental Procedures). Receptor concentration = 1.3 nM. Calculated dissociation rate constants: (●) $k_f = 3.8 \times 10^{-3}$ s^{-1}, $k_s = 3.3 \times 10^{-4}$ s^{-1}; (○) $k_s = 3.2 \times 10^{-4}$ s^{-1}. (C) Receptor adsorbed to HAP. [^{3}H]Estradiol–receptor complexes were equilibrated at 0 (●, □) or 28 °C (○) for 0.5 h and adsorbed onto HAP. (□) Complexes heated at 28 °C for 0.5 h after adsorption onto HAP. After the samples were washed with TD buffer, dissociation rates at 28 °C were measured. Receptor concentration = 0.7 nM. Calculated dissociation rate constants: (●) $k_f = 2.7 \times 10^{-3}$ s^{-1}, $k_s = 3.2 \times 10^{-4}$ s^{-1}; (○) $k_s = 2.2 \times 10^{-4}$ s^{-1}; (□) $k_s = 2.3 \times 10^{-4}$ s^{-1}. (E) Monomeric receptor. Receptor monomers were generated by treatment with 0.4 M KCl and adsorption onto HAP. After the samples were washed with TD buffer plus 0.4 M KCl, they were reequilibrated with [^{3}H]estradiol in TD buffer at 0 (●) or 28 °C (□) for 1.5 h. Dissociation was measured at 28 °C. Receptor concentration = 0.5 nM. Calculated dissociation rate constants: (●) $k_{f,monomer} = 2.5 \times 10^{-3}$ s^{-1}, $k_{s,monomer} = 3.0 \times 10^{-4}$ s^{-1}; (□) $k_{f,monomer} = 3.5 \times 10^{-3}$ s^{-1}, $k_{s,monomer} = 2.3 \times 10^{-4}$ s^{-1}. Panels B, D, and F show replots of fast-dissociating components of biphasic curves from panels A, C, and E, respectively, and were calculated by subtracting values from the extrapolated curves representing the slow-dissociating components (dashed lines) from the experimental values. The intercepts at time = 0 represent the percent contributions of the fast-dissociating components.

similar results are obtained (Figure 5C,D). Rate constants of $k_f = (2.0 \pm 0.2) \times 10^{-3}$ s^{-1} (5) and $k_s = (3.2 \pm 0.3) \times 10^{-4}$ s^{-1} (5) were found for the unheated complexes, and a single rate constant of $k_s = (2.7 \pm 0.2) \times 10^{-4}$ s^{-1} (5) was found for the heat-transformed species. These rate constants are somewhat lower than the rates obtained for receptor in solution (Figure 5A,B). Also, the percentage contribution of the fast component was much reduced, 16 ± 3% (4) as compared to 29% in solution. Apparently, interaction with the charged

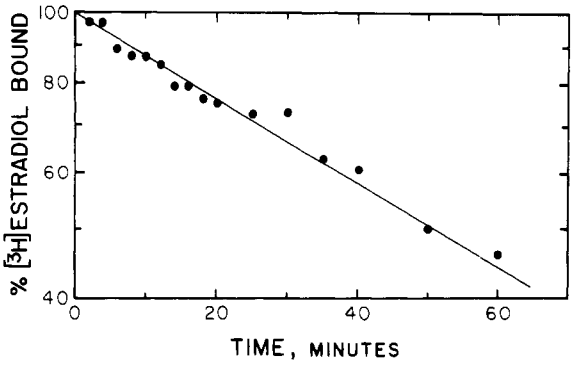

FIGURE 6: Dissociation of [^{3}H]estradiol–receptor complexes eluted from hydroxylapatite. Cytosol was equilibrated with 10 nM [^{3}H]-estradiol with or without 2 μM DES at 0 °C for 1 h. Samples were adsorbed onto HAP (2 mL) and washed 3 times with 2.5 mL of TD buffer plus 0.4 M KCl. The HAP was then extracted at 0 °C for 1 h with 0.8 mL of TD buffer plus 0.3 M sodium phosphate. The supernatant, after centrifugation at 1000g for 2 min, was diluted to 8 mL with TD buffer and allowed to sit at 0 °C for 2 h. Dissociation of [^{3}H]estradiol at 28 °C was monitored by isotopic dilution with excess DES (2 μM) as described under Experimental Procedures. Receptor concentration = 1.2 nM. Calculated dissociation rate constant: $k_s = 2.2 \times 10^{-4}$ s^{-1}.

HAP matrix promotes conversion to the high-affinity state in much the same way as elevated ionic strength (Weichman & Notides, 1979; Redeuilh et al., 1981). Heat treatment of receptor complexes preadsorbed to HAP results in complete conversion to the slow-dissociating state [$k_s = (2.8 \pm 0.2) \times 10^{-4}$ s^{-1} (5); Figure 5C]. This again indicates that transformation need not require the participation of nonadsorbed cellular components.

In low ionic strength buffer, the estrogen receptor exists as an 8S aggregate. In order to ascertain the involvement of subunit–subunit interactions in the change in steroid dissociation kinetics, the receptor was first dissociated into 4S monomers by treatment with 0.4 M KCl. This was done in the absence of estradiol to retard salt-promoted transformation. The receptor monomers were then bound to HAP, the unbound cytosol components were washed away with 0.4 M KCl buffer, and the HAP mixture was resuspended in TD buffer. Following equilibration with 5 nM [^{3}H]estradiol, dissociation was monitored at 28 °C in the presence of excess DES. As shown in Figure 5E,F, dissociation of estradiol was biphasic with dissociation rate constants of $k_{f,monomer} = (2.3 \pm 0.2) \times 10^{-3}$ s^{-1} (4) and $k_{s,monomer} = (2.7 \pm 0.3) \times 10^{-4}$ s^{-1} (4). The percentage contribution of the fast-dissociating component was 33 ± 2% (4), similar to that observed in solution (Figure 5B). This suggests that HAP-adsorbed receptor monomers are initially in a low-affinity state and are converted into a high-affinity state during the course of the dissociation assay. When the HAP-adsorbed complexes were heated at 28 °C for 40 min prior to the dissociation assay, the contribution of the fast component diminished to 23 ± 2% (5) with the dissociation rates remaining unchanged [$k_{f,monomer} = (3.0 \pm 0.5) \times 10^{-3}$ s^{-1} (5), $k_{s,monomer} = (2.2 \pm 0.0) \times 10^{-4}$ s^{-1} (5); Figure 5E,F]. No further change in this percentage was observed with incubations of up to 90 min. Thus, only about 77% of the estradiol–receptor complexes can be transformed to the high-affinity state.

The inability to transform estradiol–receptor monomers completely to the slow-dissociating state is not due to irreversible loss of transforming ability of a subset of the complexes since one would expect an increase in this fraction with prolonged incubation on HAP. This was not observed. Furthermore, receptor eluted from HAP with 0.3 M phosphate

and reequilibrated in TD buffer showed only slow dissociation kinetics [$k_s = (2.7 \pm 0.3) \times 10^{-4}$ s^{-1} (5) (Figure 6)]. We obtained this result regardless of whether unheated or heat-transformed receptors were applied to the HAP and regardless of whether or not the complexes were treated with 0.4 M KCl. It is probable that conversion to the high-affinity state in these cases was promoted by exposure to high ionic strength conditions during the elution process and by reaggregation following subsequent dilution into low ionic strength media. An obstructive effect of HAP on transformation efficiency also seems unlikely as the receptor aggregate can be completely converted to the high-affinity state while bound to HAP (Figure 5C). Thus, maintaining receptor in a monomer form limits, but does not prevent, its conversion to the high-affinity state.

The ratio of dissociation rate constants for the two monomeric states is $k_{s,monomer}/k_{f,monomer} = 0.088$ at 28 °C. This value matches that found for the receptor in solution and is evidence against additional changes in affinity state linked to receptor dimerization.

Discussion

Rat estradiol–receptor complexes which are bound to hydroxylapatite can be converted, by heating, to species whose elution profile during HAP chromatography (Figure 4) and steroid dissociation rate (Figure 5C; de Boer & Notides, 1981a) are identical with those of transformed complexes. This demonstates transformation need not require involvement of nonadsorbed cytosol components. Although we cannot absolutely rule out the interaction of HAP-adsorbed cytosol factors with the receptor, we feel it is unlikely as adsorbed receptor monomers are apparently incapable of interacting with each other (Figure 3). It remains possible that the cytosol factors proposed by others (Thrower et al., 1976; Puca et al., 1977; Thampan & Clark, 1981) are necessary for acquisition of properties of the transformed state, such as DNA binding ability or 5S formation, which we have not determined here.

Receptor monomers generated by treatment with 0.4 M KCl and adsorption onto HAP can also be transformed to the high-affinity state, if only to ≃77% completion (Figure 5E,F). Under these same conditions, the receptor's cooperative behavior is eliminated, indicating the absence of subunit–subunit interactions (Figure 3). Also, receptors eluted from HAP sediment solely as 4S species. This result, however, is likely to be a poor indicator of the state of the receptor while bound to HAP due to the ability of the phosphate elution buffer to partially dissociate dimeric receptors. It should be noted that HAP adsorption in itself, although quite tight (Figure 1), was found to have little effect upon receptor cooperativity (Figure 2) or estradiol dissociation kinetics (Figure 5C). Elimination of cooperativity only occurs when receptor is pretreated with KCl prior to adsorption, and conversion to the slow-dissociating state is strictly steroid dependent. If HAP complexed to receptor monomers in a manner which mimicked the interaction with a second receptor monomer and this was responsible for the conversion of the receptor to a high-affinity conformation, only slow estradiol dissociation kinetics would be expected. Yet, clearly, HAP-adsorbed monomers initially have fast dissociation kinetics which during the incubation with steroid turn to slow dissociation kinetics (Figure 5E). This result further demonstrates that mere dissociation of the receptor aggregate to monomers does not induce the change in affinity state.

Weichman & Notides (1977) have postulated dimerization to be essential for the transformation of receptor to the high-affinity state. Subsequent detailed study indicated the rate of conversion follows complex second-order kinetics that are very sensitive to ionic strength conditions and protein concentration (Weichman & Notides, 1979). Their conclusion that the kinetics support a dimerization mechanism must be viewed with caution in the absence of information concerning the effect of other cytosol components upon receptor kinetic properties. Our results demonstrate that partial conversion from the fast-dissociating to the slow-dissociating state occurs independent of dimer formation. Dimerization may, however, stabilize the high-affinity state as receptor which is eluted from HAP and equilibrated in low ionic strength buffer is transformed to completion (Figure 6).

Previous studies have documented the ability of monomeric 4S estradiol–receptor complexes to bind to nuclei and DNA (Sato et al., 1979; Bailly et al., 1980; Gschwendt & Kittstein, 1980; Müller et al., 1983a), also suggesting transformation is occurring in the absence of dimerization. Furthermore, Atger & Milgrom (1976) and Bailly et al. (1978) have presented evidence for an equilibrium between transformed and nontransformed glucocorticoid receptor states with generally 60–80% of the complexes capable of binding to nuclei. Transformed glucocorticoid receptors sediment as 4S monomers (McBlain et al., 1981). It is not clear, however, whether the property of nuclear- and DNA-binding ability and the property of slow dissociation kinetics are indices of the same conformational transition. Müller et al. (1983a) have found evidence for a nuclear-binding species with, in part, fast dissociation kinetics. They have recently confirmed, however, that receptor dimerization and conversion to a high-affinity state are separable phenomena (Müller et al., 1983b, 1984).

Within intact uterine cells, the estrogen receptor is distributed between nuclear and cytosolic states in an equilibrium fashion (Williams & Gorski, 1972). The nuclear-bound complexes appear to have acquired a high-affinity conformation, as indicated by a slow dissociation rate (de Boer & Notides, 1981b), as well as a dimeric form, as indicated by its sedimentation as a 5S complex (Shyamala & Gorski, 1969; Jensen et al., 1972). Regardless of the dose of estrogen administered, however, a significant fraction (10–20%) of the complexes remains cytosolic (Williams & Gorski, 1972) and 4S (Gannon et al., 1976). The equilibrium binding of estradiol to intact cells shows no evidence of cooperativity (Williams & Gorski, 1974). In contrast, transformation is very efficient (Jensen et al., 1971; Weichman & Notides, 1977) and cooperativity quite apparent (Sasson & Notides, 1983) during in vitro incubations. Thus, the in vivo behavior of the receptor better matches that predicted for receptor monomers than for receptor which is free to aggregate.

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Calculation of Free-Mg^{2+} Concentration in Adenosine 5′-Triphosphate Containing Solutions in Vitro and in Vivo†

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ABSTRACT: We have attempted to resolve the differences between the levels of free Mg^{2+} in muscle calculated by Wu et al. [Wu, S. T., Pieper, G. M., Salhany, J. M., & Eliot, R. S. (1981) *Biochemistry 20*, 7399–7403] (2.5 mM in guinea pig heart) and by Gupta and Moore [Gupta, R. K., & Moore, R. D. (1980) *J. Biol. Chem. 255*, 3987–3993] (0.6 mM in frog *skeletal* muscle) on the basis of substantially identical measurements by ^{31}P NMR of the phosphate peaks in the spectrum of $MgATP^{2-}$. The differences depend on the methods of calculation, including which reactions in which multiple equilibria are being considered. Biochemists and physical chemists customarily use different working definitions of the stability constant for $MgATP^{2-}$ in particular. Wu et al. used in their calculations, without reconciliation, methods involving three different operational definitions of the chelation equilibria involved. An algorithm for calculating Mg^{2+} and total ATP, which can be carried out with a hand calculator, is described here. With it, we calculated Mg^{2+} levels that agree with those determined by Gupta et al. [Gupta, R. K., Benkovic, J. L., & Rose, Z. B. (1978) *J. Biol. Chem. 253*, 6165–6171] with their in vitro systems. We therefore agree with the finding of Gupta and Moore that the Mg^{2+} level in skeletal and cardiac muscle is 0.6 mM.

Recently, Wu et al. (1981) published a determination of free Mg^{2+} in perfused guinea pig heart based on the known shift in the ^{31}P NMR spectrum of ATP due to Mg^{2+}. They calculated a Mg^{2+} level of 2.5 mM, in disagreement with the value of 0.6 mM found by Gupta & Moore (1980) for skeletal muscle in frog. Since the NMR spectra reported by both groups were nearly identical, this disagreement arises from different interpretations of the data. Gupta and Moore used

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