Transformation of the 8–9 S Molybdate-stabilized Estrogen Receptor from Low-affinity to High-affinity State without Dissociation into Subunits*

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The rate of dissociation of labeled estradiol from [3H]estradiol-8–9 S receptor complexes ([3H]E2–8–9 S ER) molybdate-stabilized was determined in the presence of either an excess of unlabeled hormone ("chase") or of charcoal/dextran suspension ("stripping").

Biphasic dissociation of the hormone was observed in both cases, but the fraction of the fast-dissociating component was dramatically reduced (5% instead of 60%) when stripping was used. As the dissociation patterns were independent of the degree of saturation of the receptor, the results do not favor the possibility of cooperative effects between binding sites in the 8–9 S ER.

After pretreatment of cytosol by charcoal at 28 °C for 15 min, the dissociation studied by chase displayed only the slowly dissociating component (t1/2 ~ 65 min). This effect was dependent on temperature and influenced by the ligand bound to 8–9 S ER, being pronounced with estradiol (E2) and absent with [3H]4-hydroxytamoxifen. The slow-dissociating component obtained after charcoal treatment was reconverted to fast-dissociating state by adding dithiothreitol or by incubation with cytosol at 20 °C. The charcoal treatment did not change the sedimentation coefficient (–9 S) and the Stokes radius (7 nm) of the [3H]E2–8–9 S ER, and the slow-dissociating form obtained did not bind to DNA-cellulose either in the presence or absence of molybdate ions. Thus there are likely small but functionally significant changes of structure in the 8–9 S ER which remain in a non-DNA-binding form, whereas the rate of estradiol dissociation is modified.

Steroid hormone action in target tissues is mediated by intracellular, hormone-specific receptor proteins. After binding of the hormone the receptor acquires an increased affinity for nuclear components (DNA in particular).

Buffers—Buffer A was 10 mM potassium phosphate, 12 mM α-thioglycerol, 10% glycerol, 20 mM sodium molybdate, pH 7.5 at 25 °C. Buffer B was buffer A without glycerol.

Preparation of [3H]E2-ER Complexes—Calf uteri were homogenized at 0–4 °C with a Braun mixer in 3 volumes of buffer A, with or without molybdate. The homogenate was centrifuged at 105,000 g for 1 h, and the supernatant referred to as "cytosol." Receptor binding activities and protein concentration, determined as previously described (15), were 6–8 pmol/ml and 8–10 mg/ml, respectively. The cytosol was incubated with [3H]E2 (60 Ci/mmol; CEA, France) or with [3H]4-hydroxytamoxifen (26 Ci/mmol; Amersham Corp., Buckinghamshire, United Kingdom) at a concentration of 10 nM for 17 h at 0 °C in order to label the ER-binding sites. Nonspecific binding was determined in a parallel incubation in the presence of a 100-fold excess of unlabeled E2.

Measurement of the Rate of Dissociation of [3H]E2-ER Complexes—The samples containing [3H]E2-ER complexes were equilibrated at 28 °C (5 min). Dissociation of [3H]E2 was initiated by addition of nonradioactive E2 to a final concentration of 3 μM (chase) or by adding 1/10 volume of charcoal (5%)/dextran (0.5%) suspension (stripping) with shaking. Stability control was always carried out simultaneously by keeping an aliquot of [3H]E2-ER cytosol at 28 °C (without adding either E2 or charcoal/dextran suspension). In chase experiments and stability controls, 0.2-ml aliquots were removed at various times and added to an equal volume of the charcoal/dextran suspension.
suspension kept at 0 °C. In the stripping experiments, 0.2-ml aliquots of the suspension were chilled at 0 °C. After centrifugation at 3000 x g for 10 min, the radioactivity was determined in the supernatant. The nonspecific E binding was subtracted, and the results were expressed as a percentage of the values at time 0. The loss of activity of the receptor during the experiments was always <5%. The amplitude of the fast-dissociating component was calculated by exponential analysis and it was expressed as a percentage of the binding activity of the control at time 0.

Density Gradient Ultracentrifugation—Samples containing [3H]Ez-ER complexes, diluted 1:1 in buffer B, were mixed with marker enzymes and layered onto 10-35% glycerol gradients prepared using buffer B with or without 0.5 M KCl. Centrifugation was performed at 4 °C for 17 h at 217,000 x g in an SW 60 Beckman rotor. The fractions were collected and adjusted to 450 μl with water, 100-μl portions were removed for marker enzyme assays, the radioactivity was measured. Sedimentation coefficients were determined according to the method of Martin and Ames (16) using horseradish peroxidase (Sigma, type VI, 3.6 S (17), and fungal glucose oxidase (Boehringer Mannheim), 7.9 S (18), as internal standards.

High Performance Liquid Chromatography (HPLC)—HPLC analysis of estrogen receptor was carried out using a 500 x 7.5-mm TSK G-30000SW column (LKB, Sweden) housed in a cooling jacket maintained at 4 °C. The flow rate was 1 ml/min, and 0.2-min fractions were collected. The column was calibrated with the following proteins: ferritin (6.15 nm), catalase (5.2 nm), bovine serum albumin (3.6 nm), and ovalbumin (2.75 nm). V0 and V1 values were determined by filtration of dextran blue (Pharmacia) and of tryptophan, respectively.

DEAE-Sephadex Chromatography—DEAE-Sephadex (Pharmacia, Uppsala, Sweden) was packed into columns (1 cm2, bed volume 4 ml) and equilibrated with buffer A. The sample was layered onto the column and the column washed with buffer (8 bed volumes). Elution was carried out by applying 100 ml of a continuous linear gradient (0-0.5 M KCl) in buffer A (flow rate 20 ml/h).

DNA-Cellulose Binding Assay—Samples (300 μl in duplicate) containing [3H]Ez-ER complexes were added to 300 μl of DNA-cellulose or cellulose suspension and incubated for 16 h at 0 °C. After centrifugation, the supernatants were discarded and the pellets washed with 3 x 1 ml of buffer A. The pellets were resuspended in 10 ml of 0.01 M acetate buffer, pH 7.4, and adjusted to 1 ml with water and 50 μl of 1 M KCl. 300 μl of a continuous linear gradient (0-0.5 M KCl) was then added to the samples which had been preincubated without it. The dissociation of [3H]Ez from the receptor was initiated by the addition of 3 mM unlabeled estradiol (O, C, D) or by the addition (1/10 volume) of a charcoal (0.5%)/dextran (0.5%) suspension (C). At time 15 min, one half of the samples (O) was cooled at 4 °C and centrifuged at 0 °C in order to remove charcoal, and the dissociation was performed by the addition of unlabeled estradiol (C). The data are expressed as percentage of the zero time values.

RESULTS

Dissociation Kinetics

Comparison of Chase and Stripping Studies—The dissociation of the molybdate-stabilized [3H]Ez-ER complexes as studied by chase with unlabeled estradiol is illustrated in Fig. 1. In agreement with previous reports (2, 20), this process cannot be described by simple, one-component first-order kinetics but may be interpreted by a function composed of two exponential terms:

\[ R(t) = A e^{-at} + B e^{-bt} \]

where \( R(t) \) is the radioactivity remaining associated with ER at time \( t \), and \( A, B, a, \) and \( b \) are constants \((a > b)\). Until now, this has been taken to mean that a portion of the ER has already been transformed and is responsible for the slow exponential component (apparent t1/2 ~ 70 min ± 7 min; \( n = 5 \)). It has been assumed that the fast exponential component corresponds to the nontransformed receptor (2). By exponential analysis, the apparent t1/2 of the fast exponential component was estimated to be ~8 min. The relative proportions of the fast and slow components at time 0 are \( A \sim 60\% \) and \( B \sim 40\% \).

Strikingly different results were obtained by the charcoal stripping approach. Although the data can be fitted by the same formula as above, the relative proportion of the slow component was increased to \( B \sim 90 \% \), \( n = 5 \). The half-lives for the fast and slow components are t1/2,fast ~ 5-7 min and t1/2,slow ~ 132 ± 13 min, \( n = 5 \), respectively.

The different pattern of dissociation of the [3H]Ez-ER complexes (Fig. 1) observed in charcoal stripping as compared to chase experiments could be an artifact due to the method. To test this possibility, we have carried out control experiments in which the receptor (always in molybdate-containing buffer) was exposed to the charcoal suspension for a brief period of time. The dissociation kinetics study was then obtained by the chase method, after removal of charcoal by centrifugation. The effect of an incubation of the [3H]Ez-ER complexes with charcoal for 15 min at 28 °C is shown in Fig. 1: only the slow-dissociating component can be observed (t1/2 ~ 65 ± 6.5 min, \( n = 8 \)). The presence or absence of molybdate did not influence the shift of the fast/slow component ratio as a result of charcoal treatment at 28 °C (Fig. 2). It can be concluded that the treatment of the cytosol with the charcoal/dextran suspension caused a change in the ER structure such that the rate of ligand dissociation became ~8 times slower. The ~2-fold difference between the rates of dissociation of the slow component as measured in the chase versus stripping experiments, however, may be due to the inability of charcoal to prevent total rebinding of the dissociated [3H]Ez molecule. It has to be noted that the dissociation of the transformed (5.6 S) [3H]Ez-ER complex proceeds according to the first-order kinetics, with t1/2 ~ 70-80 min close to that determined for the slow compound of the charcoal-treated, nontransformed (8-9 S) [3H]Ez-ER complexes.

Effect of Temperature and Ligand on the Fast → Slow Component Conversion by Charcoal Treatment—The effect of charcoal was temperature-dependent and was much less pronounced at 0 °C (Fig. 3). In addition, incubation of the unoccupied receptor with charcoal for 15 min at 28 °C, followed
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FIG. 2. Dissociation of [3H]estradiol from receptor in the absence of molybdate. Cytosol (in buffer A without molybdate) containing complexes was treated or not (control) with the charcoal/dextran suspension prior to dissociating assays, initiated by the addition of unlabeled estradiol. ○, control; □, sample treated for 30 min at 28 °C in the presence of charcoal/dextran suspension (1/10 volume); ■, sample treated for 30 min at 28 °C in the absence of charcoal. Results were expressed as in Fig. 1.

FIG. 4. Influence of the ligand on the charcoal treatment. Cytosol (in buffer A) containing ER was untreated or treated with charcoal for 15 min at 28 °C. [3H]E2 was then added (10 nM) and the samples were incubated for 2 h at 0 °C. The dissociation of the labeled ligand was initiated by addition of unlabeled estradiol. Results are expressed as in Fig. 1.

FIG. 3. Influence of the temperature on the charcoal treatment. Cytosol (in buffer A) containing [3H]E2-ER complexes was untreated (○) or treated (□) with charcoal for 30 min at 0 °C. [3H]E2 was then added (10 nM) and the samples were incubated for 2 h at 0 °C. The dissociation of the labeled ligand was initiated by addition of unlabeled estradiol. Results are expressed as in Fig. 1.

FIG. 5. Dissociation of [3H]4-hydroxytamoxifen from the molybdate-stabilized receptor. Cytosol (in buffer A) was equilibrated with 10 nM of [3H]4-hydroxytamoxifen for 2 h at 0 °C. Prior to the dissociation assays, samples were incubated for 30 min at 0 °C (○); or 30 min at 28 °C (■) and 30 min at 28 °C in the presence of the charcoal/dextran suspension (1/10 volume) (□). The dissociations were initiated by addition of 3 μM unlabeled estradiol. Results are expressed as in Fig. 1.

Reversibility of the Charcoal-induced Fast → Slow Component Conversion

As shown in Fig. 6, the [3H]E2-ER complex as obtained after charcoal treatment can be reconverted to the more rapidly dissociating state after incubation with added (untreated) cytosol at 20 °C for 2 h. Only a slight effect was observed after addition of charcoal-treated cytosol. The reconversion was temperature-dependent, and no effect was observed after incubation at 4 °C (data not shown).
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these results. HPLC data also confirmed that the nontransformed receptor was homogeneous in respect to its molecular size: a well-defined, symmetrical peak of ~7 nm was obtained, irrespective of charcoal exposure (Fig. 8).

DEAE-Sephadex Chromatography

The nontransformed [3H]E2-ER complexes stabilized by sodium molybdate were eluted from DEAE-Sephadex as a single peak at 0.24 M KCl (Fig. 9). Under the same experimental conditions, the [3H]E2-ER complexes treated with the charcoal/dextran suspension for 30 min at 28 °C also eluted as a single peak at a slightly lower KCl concentration (0.21 M KCl). This small difference in the KCl concentration of the eluting buffer may indicate some changes in the charge distribution on the surface of the receptor protein between the original material and after treatment with charcoal.

DNA-Cellulose Binding Assay

Transformed [3H]E2-ER complexes acquire higher affinity for nuclei and DNA. We have therefore studied the binding of charcoal-treated [3H]E2-ER to DNA-cellulose (Table I). Molybdate-containing cytosol labeled with [3H]E2 was treated or not with charcoal at 28 °C for 30 min, and then incubated with DNA-cellulose for 16 h at 0 °C. In either case, only a small fraction (<5%) of added [3H]E2-ER was bound by DNA-cellulose. In contrast, 34% of the [3H]E2-ER of the cytosol heat-treated in the absence of molybdate and thereafter supplemented with molybdate was bound by DNA-cellulose under identical conditions. These results indicate that the slow-dissociating 9 S ER form obtained after charcoal treatment is unable to bind DNA-cellulose. Experiments where the samples were adjusted to 0.4 M KCl prior to further experiments supported this conclusion. In the presence of molybdate, [3H] E2-ER complexes treated 30 min at 28 °C were converted to a molecular state able to bind DNA-cellulose. The same treatment in the presence of charcoal decreased the binding of [3H]E2-5 S ER to DNA. As shown in Table I, in the absence of molybdate, only a small amount of ER was bound by DNA-cellulose after charcoal treatment.

Molecular Size

The [3H]E2-ER complexes sedimented as a sharp 9.3 S peak in low-salt molybdate-containing sucrose gradients and as a 5.3 S peak in sucrose gradients prepared in a 0.5 M KCl-containing buffer (Fig. 7). Treatment with charcoal, whether at 0 or 28 °C (in the presence of molybdate) did not influence the dissociating component was also restored when 10 mM dithiothreitol was added to charcoal-treated cytosol (Fig. 6), suggesting that oxidoreduction changes are involved in the changes of ER conformation. Oxidation of thiol groups by atmospheric oxygen in the course of contact with charcoal, however, was unlikely, since experiments performed under nitrogen gave similar results.
Fig. 9. DEAE-Sephadex chromatography. Cytosol in buffer A was equilibrated with 10 nM \([^{3}H]E_2\). Three ml of untreated cytosol (●) and 3 ml of cytosol treated with charcoal/dextran suspension for 30 min at 26 °C (□) were fractionated on DEAE-Sephadex columns as described under "Materials and Methods." Fractions of 2 ml were collected, and radioactivity of 0.1 ml portions of each fraction was determined.

**Table I**

 BINDING OF \([^{3}H]E_2\)-RECEPTOR COMPLEXES TO DNA-CELLULOSE

Cytosol containing \([^{3}H]E_2\)-ER was treated as indicated (left column). In high-salt experiments, samples were diluted to 0.1 m KCl, and in all experiments, samples were adjusted to 20 mM molybdate prior to DNA-cellulose assays. Total and DNA-cellulose-bound \([^{3}H]E_2\)-ER were measured as described under "Materials and Methods." The abbreviations used are: MoO\(_4\)\(^{-}\), sodium molybdate, 20 mM; DCC, treatment with dextran-coated charcoal at 28 °C for 30 min.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amount of [(^{3}H)]E(_2)-ER</th>
<th>Fraction bound to DNA-cellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total fmol</td>
<td>Bound %</td>
</tr>
<tr>
<td>Low-salt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MoO(_4)(^{-}), 0 °C</td>
<td>460</td>
<td>12</td>
</tr>
<tr>
<td>MoO(_4)(^{-}), DCC</td>
<td>330</td>
<td>9</td>
</tr>
<tr>
<td>28 °C, 30 min</td>
<td>374</td>
<td>129</td>
</tr>
<tr>
<td>DCC</td>
<td>544</td>
<td>70</td>
</tr>
<tr>
<td>High-salt (0.4 m KCl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MoO(_4)(^{-}), 0.4 m KCl</td>
<td>525</td>
<td>21</td>
</tr>
<tr>
<td>MoO(_4)(^{-}), DCC, then 0.4 m KCl</td>
<td>494</td>
<td>14</td>
</tr>
<tr>
<td>28 °C, 30 min</td>
<td>618</td>
<td>261</td>
</tr>
<tr>
<td>DCC</td>
<td>506</td>
<td>50</td>
</tr>
<tr>
<td>0.4 m KCl, 28 °C, 30 min</td>
<td>558</td>
<td>294</td>
</tr>
<tr>
<td>0.4 m KCl, 28 °C, 30 min, DCC</td>
<td>599</td>
<td>287</td>
</tr>
<tr>
<td>0.4 m KCl, 28 °C, 30 min, DCC</td>
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<td>25.6</td>
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**DISCUSSION**

Biphasic dissociation patterns of labeled \(E_2\) from molybdate 8-9 S ER were observed either in the presence of an excess of unlabeled hormone (chase experiments) or in the presence of a charcoal/dextran suspension (stripping). The data are compatible with the presence of two populations of the \([^{3}H]E_2\)-ER complexes, one with a short \(t_{1/2}\) (−8 min at 28 °C), and the other with a long \(t_{1/2}\) (−70 min), similar to that calculated for the \([^{3}H]E_2\)-ER complexes transformed by temperature in the absence of molybdate. When the dissociation was studied by stripping, the fraction of the fast-dissociating component was dramatically reduced (5%), as compared to what was obtained in chase experiments (60%). The same dissociation patterns were obtained independently of the degree of saturation of the receptor and of protein concentration (not shown), results which do not favor the possibility of cooperative effects among binding sites of the molybdate-stabilized 9 S receptor.

The difference between the dissociation pattern of molybdate-stabilized \([^{3}H]E_2\)-ER complexes in stripping and chase experiments was not due to inability of charcoal to prevent rebinding of the dissociated ligand. When the dissociation was studied by the chase method after treatment of cytosol by charcoal at 28 °C for 15 min, only the slowly dissociating component was obtained with a \(t_{1/2}\) ~ 65 min. Similar results have been recently published for the rat uterus (21) and chick oviduct ER (22) and the rat thymus glucocorticosteroid receptor (23).

In order to interpret the effects of the charcoal treatment on the slow/fast component ratio, several observations have to be accounted for: 1) The charcoal effect is temperature-dependent; low at 0 °C, larger at 28 °C. 2) It is strongly influenced by the ligand bound to ER: pronounced with \(E_2\), absent with 4-hydroxytamoxifen (anti-estrogen), and small in the absence of ligand. 3) The reversal of the charcoal effect can be obtained by incubation with added untreated cytosol at 20 °C (but not at 0 °C) or by addition of a high (10 mM) concentration of dithiothreitol. 4) No significant change of the molecule size of the 9 S ER could be detected as a result of the charcoal treatment. 5) The slow component of \([^{3}H]E_2\)-ER population produced by charcoal treatment does not bind to DNA-cellulose. 6) The charcoal effect on the \([^{3}H]E_2\)-ER complexes can be produced in N\(_2\) atmosphere.

It is therefore suggested that the \([^{3}H]E_2\)-ER complexes involve (a) small \(M_i\) factor(s) present in excess in the uterine cytosol and which increases the rate of dissociation of hormone from the receptor. This factor would be removed from \([^{3}H]E_2\)-6-9 S ER complexes by adsorption to charcoal, rapidly at 28 °C and slowly at 0 °C, and slowly even at 28 °C in the absence of agonist ligand. Here we are possibly dealing with a mechanism involving an oxidereduction reaction, not requiring atmosphere \(O_2\). Recently, an NADPH-dependent thiodioxidin-mediated reducing system has been identified as an activator of the glucocorticosteroid receptor, favoring the steroid-binding form in rat liver cytosol (24). Attempts of reconstitution of the fast-dissociating \([^{3}H]E_2\)-ER complex by addition of NADPH have been unsuccessful.

The small difference noted between the elution pattern from DEAE-Sephadex of the molybdate-stabilized 8-9 S \([^{3}H]E_2\)-ER complexes, whether they have been treated or not by charcoal, may be due to a change of charge distribution on the surface of the complex.

The slow-dissociating molybdate-stabilized 8-9 S receptor does not bind to DNA-cellulose. The transformation of \([^{3}H]E_2\)-ER complexes to DNA binding states induced by high salt (0.4 m KCl) at 28 °C is not inhibited by the presence of 20 mM molybdate (see also 9, 13). However, the estradiol-receptor complexes treated by charcoal/dextran prior to transformation display a low affinity for DNA-cellulose.

One might suggest that the slowly dissociating component
obtained after charcoal treatment could be an artifact due to the presence of molybdate in the medium. However, we have shown that in the absence of molybdate: 1) pretreatment of [3H]E2-ER by charcoal gave rise to a slowly dissociating component with a t1/2 ~ 75 min, similar to the value obtained for the transformed ER-ER complex; 2) the molecular size of the [3H]E2-ER is not influenced by charcoal treatment; and 3) charcoal-treated [3H]E2-ER complexes bind to DNA-cellulose with a low affinity.

It has been reported recently that the heat-induced conversion of nontransformed 8 S or 4 S [3H]E2-ER complexes immobilized on hydroxylapatite from a state of fast to another of slow E2 dissociation can occur without formation of the 5 S complex (10, 11). The lack of effect of charcoal treatment on the 4-hydroxytamoxifen-ER complex suggests that the mechanism we are studying may have a physiological role. Other differences between the complexes of ER with E2 and the anti-estrogen 4-hydroxytamoxifen have previously been reported (25–28). These studies and our results imply that the rate of dissociation of estradiol is not necessarily dependent on the other known molecular characteristics of the receptor. The transformation of the ER to a higher affinity state for the hormone and the acquisition of affinity for DNA-cellulose are not necessarily related.

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