Two Populations of the Estrogen Receptor Separated and Characterized Using Aqueous Two-Phase Partitioning†

Michael Fritsch,‡ Iain Anderson,§ Cynthia M. Leary,§ and Jack Gorski*,§

National Institutes of Health, National Cancer Institute, Laboratory of Pathology, Building 10, Room 2N212, Bethesda, Maryland 20892, and Department of Biochemistry, University of Wisconsin−Madison, 420 Henry Mall, Madison, Wisconsin 53706-1569

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ABSTRACT: Two populations of the rat uterine estrogen receptor (ER) were separated and characterized using aqueous two-phase partitioning. Countercurrent distribution of rat uterine cytosolic ER allowed rapid and efficient separation of two populations, one population partitioned preferentially into the top phase (T, $K_{obs} = 3-6$) and the other into the bottom phase (B, $K_{obs} = 0.01-0.03$). The majority of unoccupied cytosolic ER is in the T population. Upon estrogen binding and/or heating to 30 °C in vitro the T population is converted to the B population. The transition from T to B does not exclusively involve loss of heat shock protein 90 and does not alter the ligand binding ability of the steroid binding domain. Using the human ER steroid binding domain overproduced in *Escherichia coli* and the steroid binding domain generated by partial trypsinization of the rat uterine ER, we demonstrate that the characteristic distinguishing T and B populations is not localized to this domain alone but may be associated with the amino terminal half of the ER (the A/B and DNA binding domains). The T to B transition of the ER also occurs in human MCF-7 breast cancer cells upon treatment with estrogen at 37 °C.

The estrogen receptor (ER) is a nuclear DNA-binding protein that regulates the transcription of numerous genes in an estrogen dependent manner. Upon binding estrogen the ER undergoes a conformational change that is characterized by a loss in surface hydrophobicity of the carboxy terminal steroid binding domain (SBD) (Hansen & Gorski, 1986; Fritsch et al., 1992b). Estrogen binding to the receptor is necessary to activate transcription from estrogen responsive genes. The ER is also a DNA binding protein that binds to specific enhancer sequences (estrogen response elements, ERE) with high affinity (Yamamoto, 1985; Klock et al., 1987). DNA binding localizes the ER to the proper estrogen responsive gene and allosterically modulates the SBD's conformation (Fritsch et al., 1992a).

The unoccupied ER can be extracted from cells using hypotonic buffer resulting in a cytosol containing ER. The cytosolic ER represents a mixture of receptor populations with different physicochemical properties. Two or more populations of ER have been identified with respect to several characteristics, including (1) ER interaction with heat shock protein 90 (hsp90) (Grody et al., 1982; Joab et al., 1984; Catelli et al., 1985), (2) ER binding to DNA (Yamamoto & Alberts, 1972; Yamamoto, 1974; Grody et al., 1982; Golding & Korach, 1988; Denis & Gustafsson, 1989), (3) ER interaction with other proteins (Spelsberg et al., 1988; Nelson et al., 1989; Getzenberg et al., 1990), (4) ER dimerization (Notides et al., 1981; Sasson & Notides, 1982; Sakai & Gorski, 1984), (5) alterations in ER charge states (Katzenellenbogen et al., 1987), and (6) ER hormone binding affinity (Raymoure et al., 1985, 1986; McNaught et al., 1986, 1990; McNaught & Smith, 1986; Dayani et al., 1990; Nag et al., 1990). The functional significance of any of these changes in the ER remains unknown.

Aqueous two-phase partitioning (ATPP) has been used to characterize the physical properties of steroid hormone receptors (Alberga et al., 1976; Andreasen & Mainwaring, 1980; Andreasen & Gehring, 1981; Andreasen, 1982, 1983, 1987; Walter et al., 1985; Hansen & Gorski, 1985, 1986, 1989; Sodergard, 1986; Fritsch et al., 1992b; Hansen, 1994). ATPP was used to show a significant change in the surface charge of the glucocorticoid receptor (GR) when it was heat transformed in vitro (Andreasen, 1982). Our laboratory has used ATPP to study the surface properties of the rat uterine ER. These studies revealed that ligand binding or heating the ER in vitro could each, independently, alter the behavior of the ER within a given ATPP system (Hansen & Gorski, 1985, 1986, 1989; Fritsch et al., 1992b). In our efforts to further investigate the physical properties of the ER in ATPP systems we discovered the presence of two distinct populations of ER that could be characterized by different partitioning behavior. One population partitions preferentially into the upper or top phase (designated the T population) and the other into the lower or bottom phase (designated the B population).

We show in this study that the majority of the unoccupied ER is in the T population upon extraction into a cytosol. Upon binding E_2 or heating the cytosolic ER, a portion of the T population is converted to the B population. We have determined that the physicochemical difference between the T and B populations does not reside within the SBD alone and could be due to differences in the amino terminus of the ER or related to other proteins interacting with the ER.

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* Author to whom correspondence should be addressed.

‡ NIH, NCI.

§ University of Wisconsin−Madison.

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However, the transition from T to B populations is not directly related to the loss of hsp90 and does not alter the ligand binding affinity of the SBD. We also present evidence that the T to B transition occurs within the intact cell upon treatment with E_2 at 37 °C.

MATERIALS AND METHODS

17β-[2,4,6,7-^{3}H]Estradiol (E_2) (90−110 Ci/mmol) and (*Z*)-4-[*N-methyl*-^{3}H]OHT (80−100 Ci/mmol) were obtained from New England Nuclear (Boston, MA) and Amersham (Arlington Heights, IL). Immature Sprague-Dawley female rats (19 days of age) were from Harlan Sprague Dawley (Madison, WI). Bio-Gel HT hydroxylapatite (HAP) was from Bio-Rad Laboratories (Richmond, CA). Ready Safe scintillation cocktail was purchased from Beckman (Fullerton, CA). Polyethylene glycol (PEG)−palmitate and unsubstituted PEG (M_r 8000) from the same lot were obtained from Aqueous Affinity AB (Arlov, Sweden). All other chemicals were reagent grade. All procedures were performed at 4 °C unless otherwise indicated.

Preparation of Rat Uterine Cytosolic ER. Preparation of rat uterine cytosolic ER was previously described (Fritsch et al., 1992a; Murdoch et al., 1990; Hansen & Gorski, 1985). In brief, uteri from 19-day-old rats were homogenized in 10 mM Tris-HCl (pH 7.5 at 25 °C), 1.5 mM EDTA, 10 mM mercaptoethanol (TEM) buffer at 3 uteri/mL and centrifuged at 436000*g* for 10 min to obtain cytosol-containing unoccupied ER (U). The unoccupied ER was incubated with 5−10 nM [^{3}H]E_2 (EO) or [^{3}H]-4-hydroxytamoxifen (OHT) for 1.5 h at 4 °C. Heated forms were obtained by placing the unoccupied or estradiol-occupied (45 min at 4 °C) ERs at 30 °C for 45 min. The resulting ER forms were the unoccupied heated (UH) and the E_2 occupied heated (EOH). Parallel incubation of samples containing 200-fold molar excess of unlabeled DES was used to determine nonspecific binding for all ER forms.

Hydroxylapatite (HAP) Assay. The amount of ER was determined using the HAP assay as was previously described (Fritsch et al., 1992a; Murdoch et al., 1990; Hansen & Gorski, 1985).

Immunoblotting. Immunoblotting was performed as previously described (Fritsch et al., 1993, 1992a; Furlow et al., 1990). The primary antibody was affinity-purified polyclonal rabbit antibody ER715 (1:1000) raised against a synthetic peptide with a sequence derived from the D region of the rat ER (Furlow et al., 1990). The second antibody was a goat anti-rabbit IgG (1:5000) linked to alkaline phosphatase, and the substrate (BCIP/NBT) was added for color development.

Preparation of the Overexpressed Human ER SBD. The human ER SBD was overproduced in *Escherichia coli* and solublized by the method previously described (Ahrens et al., 1992; Fritsch et al., 1992b).

Aqueous Two-Phase Partitioning (ATPP). Phase systems were prepared as previously described with the following changes (Fritsch et al., 1992b). Dextran and methoxyPEG were initially stored as 20−30% (w/w) stock solutions in ddH_2O at 4 °C. Equilibrated phase systems composed of 7.67% (w/w) each of dextran and methoxyPEG with 0.167 M K_2HPO_4 (pH 7.4) in TEM were premixed. Aliquots (0.9 mL) of this polymer and salt mixture were added to 0.6 mL of buffer (TEM) and cytosol at 4 °C. The final phase system (1.5 mL final volume) consisted of 4.6% dextran, 4.6% methoxyPEG, and 0.1 M K_2HPO_4 (pH 7.4) in TEM. The final mixture of polymers, salts, and cytosol were vortexed for 10 s and then centrifuged for 5 min at 600*g* at 4 °C to form the two phases. Aliquots of each phase were sampled using a positive displacement pipet (SMI) and incubated with tritiated ligand for 1.5 h at 4 °C for the U and UH ER forms or HAP assayed immediately for the ligand-occupied ER forms. The phase H_2O content was determined as previously described (Fritsch et al., 1992b) and consisted of 90−95% H_2O for the upper phase and 80−85% for the lower phase. For affinity partitioning experiments the final phase concentration of PEG−palmitate was 10−15 μM. For [^{3}H]E_2- or [^{3}H]OHT-occupied samples partitioned in the presence of PEG−palmitate the phase systems were made 5 nM in the respective tritiated ligand to optimize ER recovery. For the unoccupied ER forms in the PEG−palmitate a similar final volume of ethanol (<0.1%) was added to the phase system. The addition of 5 nM [^{3}H]E_2, [^{3}H]OHT, or ethanol to the phase system without PEG−palmitate had no effect on the K_{obs} values (data not shown). The partition coefficients (K_{obs}) and the ER recovery were calculated exactly as previously described (Hansen & Gorski, 1985). The K_{obs} value is the ratio of the concentration of ER in the upper phase divided by the concentration of ER in the lower phase ($K_{obs} = [ER]_U/[ER]_L$).

For the studies involving molybdate, cytosols were prepared in TEM or TEM plus 20 mM Na_2MoO_4. Each ER form was then prepared and partitioned in phases made as described above but also containing 20 mM Na_2MoO_4 for both cytosol types. This was to control for any alterations in the partitioning behavior of the ER induced by the addition of Na_2MoO_4 to the phase system.

Countercurrent Distribution. Countercurrent distribution (CCD) is a method used to separate proteins with different K_{obs} values (Walter et al., 1985). CCD is analogous to simple chromatography, where each CCD tube represents a single theoretical plate. For a six-tube CCD, five blank phase systems are prepared in tubes with TEM instead of cytosol. The blank phases are separated as described above, and 90% of the blank upper phases are removed and saved, leaving the blank bottom phase within each tube (tubes CCD2 through CCD6). Cytosol is partitioned as described above for a single-tube partitioning experiment, and this tube is designated tube 1 (CCD1). Approximately 90% of the upper phase from CCD1 is removed and placed on the blank lower phase in CCD tube 2 (CCD2). Only the upper phase is transferred during CCD. This is analogous to the mobile phase in chromatography. Fresh blank upper phase is placed back on the lower phase in CCD1. Both tubes are vortexed for 10 s and spun to separate the phases. If the CCD is stopped here, this is a two-tube CCD and the upper and lower phases from each tube can be sampled to determine the K_{obs} value for each tube and the amount of ER recovered in each tube (as in Figure 3). If there are two populations of ER with differing K_{obs} values present in a cytosol, then any ER that partitions with a K_{obs} value greater than 1 will be predominantly within the upper phase that has been transferred to CCD2. We will designate this K_{obs} value as K_T, where T represents top phase preference (i.e., $K_{obs} > 1$). The population of ER that has this K_T value will be called the TOP population (T) because the ER population has a preference for the upper or top phase and will be found

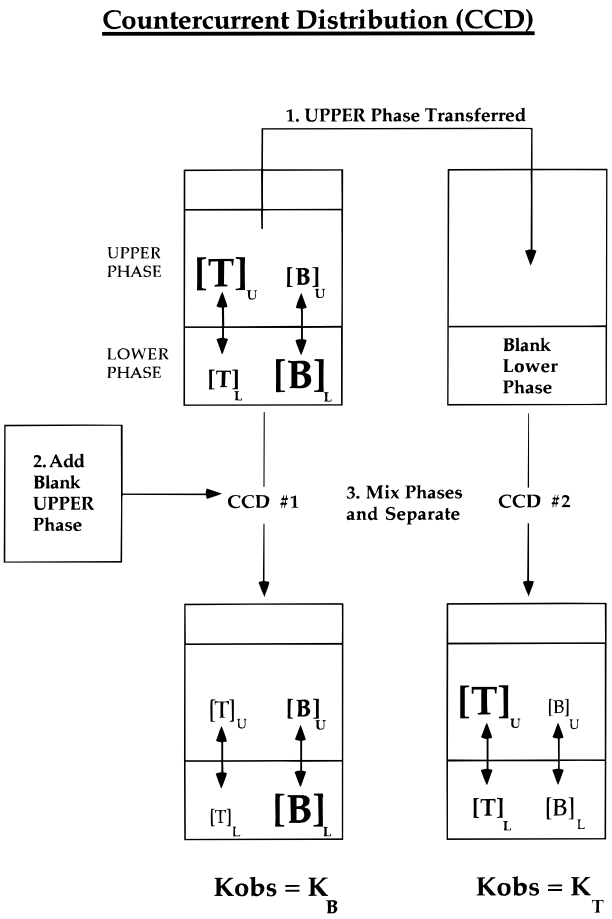

FIGURE 1: Schematic diagram of two-tube countercurrent distribution (CCD) theory for two populations of a protein that partition differentially in an ATPP system. One population partitions preferentially into the upper (top) phase, designated population T, and the other into the lower (bottom) phase, designated population B. A small amount of T will be in the lower phase and a small amount of B will be in the upper phase, depending on their K_{obs} values. Repartitioning of the upper and lower phases allows for the separation of the two populations. See Materials and Methods for more details.

predominantly in CCD2 of a two-tube CCD. Any protein that partitions with a K_{obs} value less than 1 will be predominantly within the bottom phase and will remain in tube CCD1 because only the top phase is moved. We will designate this K_{obs} value as K_B, where B represents a population of ER with a bottom phase preference (i.e., $K_{obs} < 1$) and will remain mostly in CCD1. The larger the K_{obs} value of an ER population the more of that population that will be transferred to CCD2 and similarly the smaller the K_{obs} value of an ER population the larger the amount of that protein that will remain in CCD1. Because the T population is in equilibrium between the upper and lower phases, some of the T population of ER (a K_T value greater than 1) will remain within CCD1 in the bottom phase (T population in lower phase), and similarly some of the B population of ER (a K_B value less than 1) will be transferred to CCD2 with the upper phase (B population in upper phase). When fresh blank upper phase is added to CCD1 and mixing both tubes (CCD1 and CCD2), the T and B populations will again separate within each tube with their expected K_{obs} values of K_T and K_B. For a six-tube CCD, this method of moving 90% of the upper phase from each tube to the next tube each time and CCD1 always receiving fresh blank upper phase (see Figure 1) can be continued. After the final partitioning

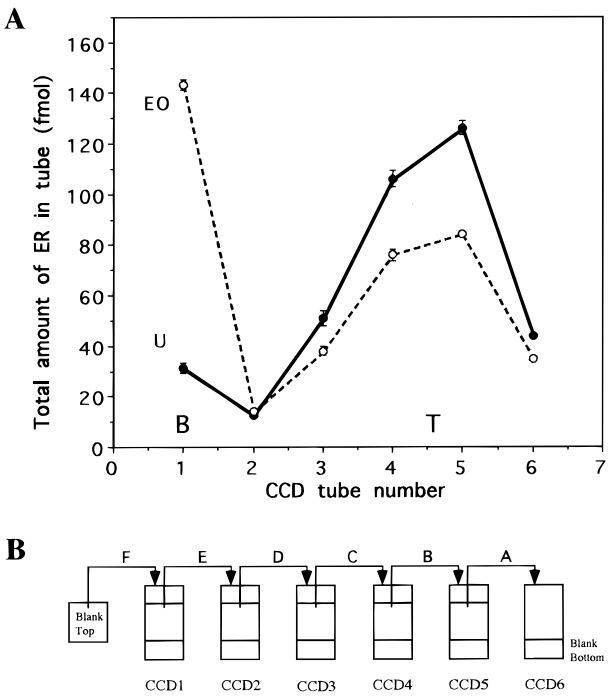

FIGURE 2: (A) CCD (six tubes) of the unoccupied ER (U, closed circles and solid line) or the E_2 occupied ER (EO, open circles and dashed line). The data are plotted as the total amount of ER recovered in each tube of the CCD (upper and lower phases combined). T represents the population of ER that partitions into the top phase (predominantly in tubes 4 and 5) and B represents the population of ER that partitions into the bottom phase (predominantly in tube 1), as determined by the K_{obs} values. The K_{obs} values for each tube of the unoccupied ER are as follows: tube 1, 0.011 ± 0.007; 2, 0.661 ± 0.097; 3, 1.398 ± 0.083; 4, 2.534 ± 0.164; 5, 4.006 ± 0.253; and 6, 6.082 ± 0.126. The K_{obs} values for each tube of the [^{3}H]E_2-occupied ER are as follows: tube 1, 0.012 ± 0.001; 2, 0.561 ± 0.05; 3, 1.171 ± 0.025; 4, 1.904 ± 0.003; 5, 2.886 ± 0.033; and 6, 4.242 ± 0.169. The percent recoveries for both ER forms were 62%. (B) Shown is the method of the final CCD for six tubes as described in the text. The upper phase from CCD5 is first transferred to CCD6 containing blank lower phase, A, then the upper phase from CCD4 is transferred to CCD5, B, etc. Finally, blank upper phase is placed onto the lower phase remaining in CCD1, F. All six tubes are mixed and centrifuged to separate the two phases, and aliquots from the upper and lower phases are sampled from each tube to determine the K_{obs} values and the total amount of ER in each tube (CCD1 through CCD6).

the K_{obs} values for each tube of a CCD can be determined as described above (see Figure 2B).

If there is a single population of ER and CCD is performed, a single peak of [^{3}H]E_2 binding activity (ER) will occur in a plot of [^{3}H]E_2 binding versus CCD tube number. Each CCD tube containing ER should have the same K_{obs} value. Where the peak of [^{3}H]E_2 binding activity occurs in a given CCD depends upon the K_{obs} value of the ER and how many tubes are used in a CCD.

If there are two populations of ER with significantly different K_{obs} values and CCD is performed, two distinct peaks of [^{3}H]E_2 binding activity will occur in a plot of [^{3}H]-E_2 binding activity versus tube number (see Figure 2A). On the basis of the findings within this paper rat uterine cytosolic ER consists of a mixture of two populations of ER. We designate one population T, for top phase preference, which has a K_{obs} value of 3.5 (determined experimentally; see Figure 2). If a pure T population of ER is partitioned over and over, it will always separate with a K_{obs} value of 3.5. We

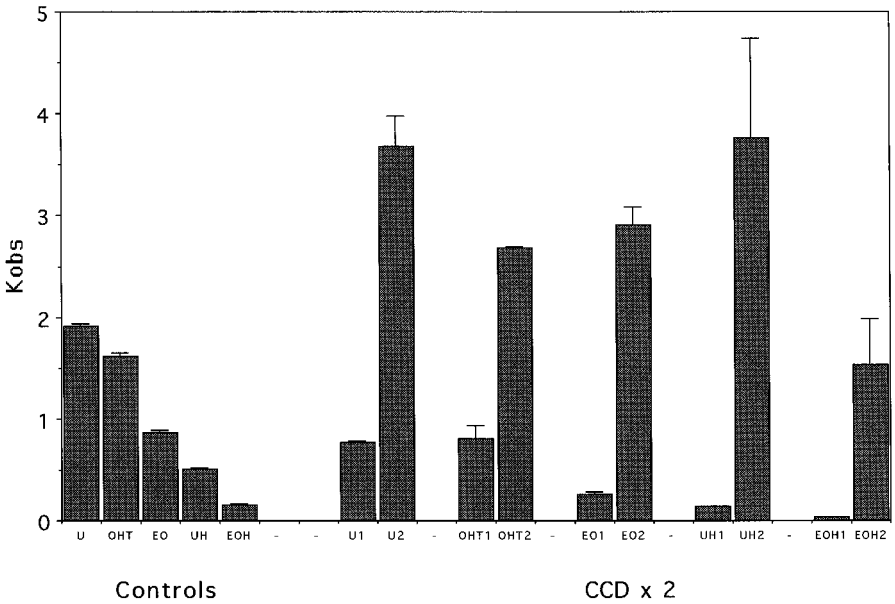

FIGURE 3: CCD (two tubes) for five different forms of the rat uterine cytosolic ER. The controls represent single tube partitioning values for each form of the ER [unoccupied (U), OHT-occupied (OHT), E_2-occupied (EO), unoccupied-heated (UH), E_2-occupied-heated (EOH)]. CCD × 2 represents a two-tube repartitioning of the cytosol. Tube 1 represents the initial tube used for partitioning, and tube 2 represents the tube into which the top phase was transferred. Percent recoveries ranged from 52% to 76%.

designate the other population of ER as population B, for bottom phase preference, which has a K_{obs} value of 0.01−0.03. If there is a mixture of T and B populations of ER within rat uterine cytosol, the K_{obs} value of a single-tube partitioning depends upon the absolute K_{obs} value of each population and the amount of each population and can be calculated by eq 4. In order to separate the two populations of ER, CCD is performed. Since the T population is predominantly in the upper phase, most of it will be moved to the next tube and since most of the B population will be in the lower phase it will remain in the initial tube. However, a certain amount of B will be transported with T in the upper phase transferred to CCD2 and a certain amount of T will remain in the lower phase in CCD1. After repartitioning, each ER population will again partition with a K_{obs} value of 3.5 for T and 0.01−0.03 for B. Depending upon the number of tubes in a CCD, most of the T population will elute as a peak in the later tubes and most of the B population will remain in the initial tubes (see Figure 2A).

Calculating the Predicted K_{obs} Value for Each ER Form. For Figure 3, the predicted K_{obs} values for each CCD tube were determined in the following manner. Based on the assumption that there are two populations of ER, one called T (top) that preferentially partitions into the upper phase with a K_{obs} value of $K_T = 3.5$ and one called B (bottom) that preferrentially partitions into the lower phase with a K_{obs} value of $K_B = 0.03$, the K_{obs} value for any tube containing a mixture of T and B can be determined if the amount of each ER population is known. The values of $K_T = 3.5$ and $K_B = 0.03$ were determined experimentally from Figure 2 and other studies (data not shown). Therefore, for any ER form (U, UH, EO, EOH, OHT) a single-tube K_{obs} value is determined by the amounts of T and B populations in the mixture (see eq 4). The amount of T and B ER populations in each ER form can be determined using the measured K_{obs} value for a single-tube partitioning and solving the following four equations:

$$TOT = T_U + T_L + B_U + B_L \quad (1)$$

where TOT = total amount (in moles) of ER measured within a tube (T and B populations inclusive), T_U = amount of T ER population in the upper phase (in moles), T_L = amount of T ER population in the lower phase (in moles), B_U = amount of B ER population in the upper phase (in moles), and B_L = amount of B ER population in the lower phase (in moles);

$$K_T = [T]_U/[T]_L \text{ (definition of a } K_{obs} \text{ value)} \quad (2)$$

where K_T is assumed to be 3.5 (determined experimentally) $[T]_U$ = concentration (moles/liter) of T ER population in upper phase, and $[T]_L$ = concentration (moles/liter) of T ER population in lower phase;

$$K_B = [B]_U/[B]_L \quad (3)$$

where K_B is assumed to be 0.03 (determined experimentally), $[B]_U$ = concentration (moles/liter) of B ER population in upper phase, and $[B]_L$ = concentration (moles/liter) of B ER population in lower phase; and

$$K_{obs} = ([T]_U + [B]_U)/([T]_L + [B]_L) \quad (4)$$

The K_{obs} value is calculated directly for the single-tube partitioning and for each CCD tube by measuring the amount of ER in each phase. Also known are the volumes of the upper phase (0.94 mL) and the lower phase (0.56 mL). The volume of the upper phase transferred during CCD was 0.82 mL. Therefore, the amount of T_U, T_L, B_U, and B_L can be determined for the singly partitioned control tube. Since the volume of upper phase transferred to CCD2 is known, the amount of T and B populations transferred to CCD2 is known $\{[T]_U(0.82\text{ mL}) = T_{U(transferred)}$ and $[B]_U(0.82\text{ mL}) = B_{U(transferred)}\}$. Since $B_{U(transferred)}$ and $T_{U(transferred)}$ will partition on the basis of their K_{obs} values (K_B and K_T), we can now solve two additonal equations:

$$B_{U(transferred)} = B'_U + B'_L \quad (5)$$

where B'_U = the amount of B in the upper phase in CCD2 and B'_L = the amount of B in the lower phase in CCD2, and

$$T_{U(\text{transferred})} = T'_U + T'_L \quad (6)$$

where T'_U = the amount of T in the upper phase in CCD2 and T'_L = the amount of T in the lower phase in CCD2.

The concentration of each ER population in CCD1 and CCD2 can be determined, and from eq 4 the predicted K_{obs} value for each tube can be calculated. Similarly the percentage of each ER population in each tube can be determined from T_U, T_L, B_U, and B_L (Walter et al., 1985).

Saturation Binding Analysis. Aliquots of rat uterine cytosolic ER were partitioned by CCD to three tubes for the unoccupied ER (U) and two tubes for the unoccupied heated ER (UH). The lower phases were collected from CCD1, which contain the bottom ER population and the upper phases were collected from CCD2 (UH) or CCD3 (U), which contain the top population of ER. An aliquot of each population from each ER form was diluted into a mixture of TEM buffer and opposite blank phase, such that the final ligand incubation solution contained an identical amount of PEG, dextran, salts, and buffer for each population. [^{3}H]E_2 was added last to make the appropriate concentration of ligand (0.05−10 nM). The final assay volume varied (3−4.5 mL) with different experiments, however, the volumes were constant within any given experiment, and parallel tubes containing 200-fold excess unlabeled diethylstilbestrol (DES) were used to determine nonspecific binding. The final dilution of cytosol was greater than 30-fold. The tubes were incubated at 4 °C for 2 h. Each sample had 500 μL of 70% HAP added for 30 min at 4 °C with several mixings. The HAP was pelleted, and an aliquot (150 μL) of the supernatant was removed and placed directly into a scintillation vial with 4 mL of scintillation fluid to directly determine the concentration of free [^{3}H]E_2. The HAP pellets were then processed as usual, and the amount of specific [^{3}H]E_2 bound was determined. The equilibrium dissociation constant (K_d) was estimated by a nonlinear least squares fit of the data using the LIGAND computer program (Munson & Rodbard, 1980).

Sucrose Density Gradient Analysis. The cytosolic rat uterine ER was occupied with 10 nM [^{3}H]E_2 ± 200-fold excess DES at 4 °C for 2 h and partitioned. A 280 μL aliquot of the [^{3}H]E_2-occupied ER from the upper phase was mixed with 40 μL of dansylated albumin (6 mg/mL) as a molecular mass standard, and 260 μL of this mixture was loaded on a 7%−20% gradient of sucrose in TEM buffer. The sucrose gradient was prepared by placing 1.2 mL of 20%, 16%, 11.5%, and 7% sucrose solutions consecutively on top of each other and allowing this step gradient to sit at 4 °C for 24 h. The gradients were centrifuged in a Beckman ultracentrifuge at 4 °C at 42 000 rpm for 14 h in an SW 50.1 rotor. Approximately 30 fractions (170 μL/fraction) were collected per gradient tube by puncturing the bottom of the tube. The peak fraction for the internal standard (dansylated albumin) was identified visually using UV light to identify the most intense fluorescence. Each fraction was processed using the HAP assay. Specific binding was calculated by subtracting the dpms from the fractions containing [^{3}H]E_2 + 200-fold excess DES from the dpms of the fractions containing [^{3}H]E_2 only. For the UH ER, the unoccupied cytosol was heated and partitioned and an aliquot of the upper phase was made 10 mM in Na_2MoO_4, in order to prevent further transformation (loss of hsp90) during the centrifugation, and placed directly on the sucrose gradient with dansylated albumin. The same results were obtained without adding molybdate (data not shown). The fractions from the sucrose gradient were incubated at 10 nM [^{3}H]E_2 ± 200-fold excess unlabeled DES for 1.5 h at 4 °C. The fractions were processed by HAP assay, and the specific dpms determined as described above.

Cell Culture. MCF-7 breast cancer cells were passaged 85−95 times in phenol red-free DMEM with 5 mM HEPES, 44 mM sodium bicarbonate, 10% bovine calf serum, 1 μg/mL of insulin, and antibiotics (100 units/mL of penicillin G-sodium, 100 μg/mL of streptomycin sulfate, and 0.25 μg/mL of amphotericin B as Fungizone). The cells were split 1:10 on each passage, which was every 7 days. For individual experiments the cells were cultured for 7−8 days in 100 mm plates, fed every 3−4 days, and always fed 24 h before harvesting. This cell line has about 150 000 ER per cell (unpublished data). ER was occupied in situ by adding fresh medium containing 8 nM [^{3}H]E_2 ± 200-fold excess unlabeled DES to the plates. For the unoccupied ER fresh medium containing ethanol (final [ethanol] < 0.1%) was added. The unoccupied plates were allowed to sit at 37 °C for 60 min. The plates designated O2 were incubated either at room temperature or 37 °C (this did not have any effect on the K_{obs} value) with [^{3}H]E_2 for 2 min and then placed on ice for 58 min. The plates designated O60 were incubated with [^{3}H]E_2 for 60 min at room temperature or 37 °C. The cells from all groups were rinsed well with Hank's balanced saline solution, scraped into TEM buffer at 4 °C, homogenized with 30 strokes in a Dounce homogenizer with a B pestle, allowed to sit 5−10 min, and then homogenized with 10 more strokes. The extract was then made 0.4 M in K_2HPO_4 (pH 7.4) for 30 min at 4 °C with occasional mixing. The extract was centrifuged in a fixed angle rotor (TLA-100.2) at 436000*g* for 10 min in a Beckman tabletop TL-100 ultracentrifuge at 4 °C. The resulting supernatants (whole cell extracts) for each ER form (unoccupied, O2, and O60) were partitioned. The unoccupied ER was occupied and/or heated in vitro as for the rat uterine cytosolic ER to generate the other ER forms UH, EO, and EOH. The O2 and O60 extracts were heated to 30 °C for 60 min in vitro to generate the O2H and O60H samples. Aliquots were partitioned exactly as described for the rat uterine cytosolic ER in the same ATPP phase system. The final K_2HPO_4 concentration within the phases was kept at 0.1 M by adjusting for the amount of K_2HPO_4 in each whole cell extract.

In order to demonstrate that the ER was occupied in situ and not during extraction the following experiment was performed. Cells were labeled exactly as above except that the TEM used for harvesting the cells was made 2 μM in DES to occupy any unoccupied ER during extraction. The amounts of ER recovered under these conditions were U, 484 (control, no DES); O2, 523; and O60, 504 fmol of ER/mg of protein, indicating that the ER was completely occupied under the in situ conditions described above. The partition coefficients were exactly the same as for the ER harvested in TEM without DES.

RESULTS

Identification of Two Populations of ER within Rat Uterine Cytosol. Previous reports from this laboratory using ATPP showed that the unoccupied ER partitioned differently from the E_2 occupied ER (Hansen & Gorski, 1985, 1986). In order to characterize the nature of these ATPP-detected differences between the unoccupied and E_2 occupied rat uterine cytosolic ERs we employed the method of countercurrent distribution (CCD) with the ATPP system (Walter et al., 1985). Shown in Figure 1 is a schematic diagram illustrating the method of two-tube CCD. CCD allows one to determine whether a protein partitions as a homogeneous single population within the ATPP system or as more than one population (see Materials and Methods). Using CCD to six tubes (see Figure 2), we separated two populations of the ER protein present in both [^{3}H]E_2-occupied ER and unoccupied ER from rat uterine cytosol, one population preferring to partition into the upper or top phase (designated T) with a K_{obs} value of 3.5 (see Figure 2 legend, tubes 4 and 5) and one preferring to partition into the lower or bottom phase (designated B) with a K_{obs} value of 0.01−0.03 (see Figure 2 legend, tube 1). These two populations can be rapidly and efficiently separated using CCD. Rat uterine cytosol containing unoccupied ER (U) was partitioned into CCD tube 1 (CCD1) as described in Materials and Methods. Ninety percent of the top or upper phase from CCD1 was placed on blank bottom or lower phase (the tube also contained 10% blank top phase) in CCD2, while an equal volume of fresh blank upper phase was placed back into CCD1 containing the bottom phase from the original unoccupied cytosol partitioning. CCD1 and CCD2 were mixed, and the phases were separated by centrifugation. The upper phase (90%) from CCD2 was placed on fresh blank bottom phase in CCD3, while the upper phase from CCD1 was transferred to CCD2 and fresh blank upper phase was again placed on the lower phase in CCD1. These tubes were mixed and the phases separated. This procedure was continued for a total of six times with the final tube (CCD6) containing predominantly upper phase from the original CCD1 and the original lower phase of CCD1 remaining entirely in CCD1. Each transfer involves moving only upper phase from each tube to the next lower phase and CCD1 receiving fresh blank upper phase onto the original bottom phase for each partitioning. The final transfer is illustrated in Figure 2B. After CCD to six tubes was completed, the K_{obs} values for each of the six tubes (CCD1 to CCD6) were determined by sampling both the upper and lower phases and determining the specific [^{3}H]E_2 in each phase as described in Materials and Methods. The K_{obs} value for each tube was calculated and reported in the legend for Figure 2. This allows one to determine whether there are one or two populations of ER in cytosol. If there was a single population of ER separated by CCD, then a plot of [^{3}H]E_2 binding activity versus CCD tube number should have shown a single peak of [^{3}H]E_2 binding activity and each tube with ER would have the same K_{obs} value. If there were two populations of ER separated by CCD with different K_{obs} values, then a similar plot should have shown two peaks of [^{3}H]E_2 binding activity as we observed in Figure 2.

Figure 2 shows the results after six-tube CCD, plotted as the total amount of ER in each CCD tube versus the CCD tube number. Clearly there are two populations (peaks) for both the unoccupied and the [^{3}H]E_2-occupied ER within a cytosol. The solid line represents the unoccupied rat uterine cytosolic ER (U) which consists of a small amount of population B in CCD1 and a large amount of population T predominantly in tubes CCD4 and CCD5. The ER was occupied with [^{3}H]E_2 at 4 °C (EO) prior to CCD and the same two populations were observed, as determined by the K_{obs} values for each tube (see Figure 2 legend), but the quantity of the B population (CCD1) was much larger for the [^{3}H]E_2-occupied ER than for the unoccupied ER and the amount of the T population (CCD4 and CCD5) was correspondingly reduced (open circles, dashed line). This would explain the differences for the single tube K_{obs} values previously reported (Hansen & Gorski, 1985, 1986; Fritsch et al., 1992b). The occupied ER has more B ER, and thus the single-tube K_{obs} (an average K_{obs} for both populations in a single tube) is lower than for the unoccupied ER. The K_{obs} value of the B population is about 0.01−0.03 and for the T population it is between 3.5 and 6, as determined experimentally from the measured K_{obs} values reported in the Figure 2 legend and numerous other experiments (data not shown). There is some difference in the actual K_{obs} values between the unoccupied and occupied T populations that could represent subtle differences between the two T populations (see Figure 2 legend, K_{obs} values for tubes 5 and 6). However, there are at least two distinct populations of ER in a rat uterine cytosol that can be distinguished by CCD in an ATPP system. Binding of [^{3}H]E_2 to the ER prior to separation within the ATPP CCD system converts a portion of the T population to the B population. Since the K_{obs} values of the two populations (T and B) differ by almost a factor of 400, the two populations can be rapidly and efficiently separated by a simple two-tube CCD. Therefore, with only a two-tube CCD most of the B population (small K_{obs}) will be in CCD1 and most of the T population (large K_{obs}) will be in CCD2.

Characterizing the Properties that Distinguish the Two Populations. In order to characterize the different properties between these two populations of ER, the effects of ligand binding and heating were studied (Figure 3). In Figure 3 the K_{obs} values for the controls, which are determined from single tube partitionings (i.e., cytosol treated as indicated, partitioned once in the ATPP system, followed by determination of the K_{obs} values), demonstrate that the K_{obs} value decreases only slightly from the unoccupied ER value when the ER is occupied prior to partitioning with the antiestrogen 4-hydroxytamoxifen (OHT), but decreases more when the ligand is E_2 (EO). Heating the unoccupied (UH) or E_2 occupied (EOH) cytosol prior to partitioning reduced the K_{obs} values much more. Heating the cytosolic ER traditionally has resulted in changes in its physicochemical properties, a process called transformation (Grody et al., 1982), which includes the loss of heat shock protein 90 (hsp90).

The two-tube CCD of each of these forms of the ER is also shown. There were at least two populations of ER present for each form of ER, as evidenced by the very different K_{obs} values determined for each of the two CCD tubes. The K_{obs} value for each tube and each ER form (U, OHT, EO, UH, EOH) is determined by the absolute amount of each population (T and B) present in that tube. A large amount of the T population will result in CCD2 containing predominantly pure T population with a K_{obs} value of about 3.5 (see control U, Figure 3) and in CCD1 enough of the T

population will be left behind to result in a K_{obs} value in CCD1 much greater than the expected 0.01−0.03 (see U1 and U2, Figure 3). Similarly, if the majority of the ER is in the B population (see control EOH, Figure 3), the K_{obs} value of CCD1 will be close to the expected 0.01−0.03 and the K_{obs} value of CCD2 will be less than the expected 3.5 value because a proportion of the ER in CCD2 will be B population ER that was present in the top phase in CCD1 prior to transfer (see EOH1 and EOH2, Figure 3). The predicted K_{obs} value for each tube (CCD1 and CCD2) for each ER form can be calculated (see Materials and Methods). The measured K_{obs} values for CCD1 and CCD2 for each ER form are almost exactly as predicted assuming the presence of two populations, one with a K_{obs} of 3.5 and one with a K_{obs} of 0.03 (see Materials and Methods). It should be noted that the partitioning behavior of each population is determined by qualitative characteristics of the ER. Although the measured K_{obs} is mathematically defined as $[ER]_U/[ER]_L$, many factors determine how a protein partitions in a given ATPP system. The factors affecting the partition coefficient (K_{obs}) of a protein are defined by the following equation (Walter et al., 1985; Hansen & Gorski, 1985):

$$\ln K_{obs} = \ln K_o + (ZF/RT)\,\Delta\Psi \qquad (7)$$

where Z is the net molecular charge on the protein and $\Delta\Psi$ is the salt-dependent interfacial potential difference, which is constant for a given phase system. The other terms are Faraday's constant (F), the gas constant (R), and temperature (T). K_o is the contribution to the partition coefficient due to nonelectrostatic surface interactions of the protein with the phase environment.

We calculated the predicted K_{obs} values for each CCD tube as described in Materials and Methods. These calculations are based on the assumption that all the ER was in one of the two populations, T (K_{obs} 3.5) or B (K_{obs} 0.03), as well as using the measured K_{obs} values from the single-tube partitioned controls in Figure 3. The predicted K_{obs} values are as follows: U1, 0.7, U2, 3.3, EO1, 0.27, EO2, 2.9, UH1, 0.13, UH2, 2.6, EOH1, 0.03, and EOH2, 1.6, and these are essentially the same as the measured values (Figure 3) within experimental error. The percentages of the ER in the T and B populations for each ER form (any ER form represents a mixture of T and B) were also calculated. The percentages of ER in the T population were 89% for the unoccupied ER, 85% for OHT-occupied ER, 67% for the E_2-occupied ER, 51% for the unoccupied heated ER, and 19% for the E_2-occupied heated ER. The remainder of the ER was in the B population. Clearly the differences observed in the single-tube K_{obs} values as a function of ligand and heating are due to differences in the quantity of each of the two populations present in each ER form. Although CCD can be used to separate the two populations, the change in the single-tube K_{obs} values can also be used as a very rapid assay for monitoring the T to B transition. Estrogen apparently leads to the conversion of some of the ER from T to B, whereas OHT has only minimal effects. Heating the cytosolic preparation to 30 °C for 45 min enhances the conversion of T to B, and the combination of E_2 and heating drives the transition even further toward the B population. This transition from T to B is probably irreversible at 4 °C (Hansen & Gorski, 1986, 1989). The underlying properties of the T to B transition are not known.

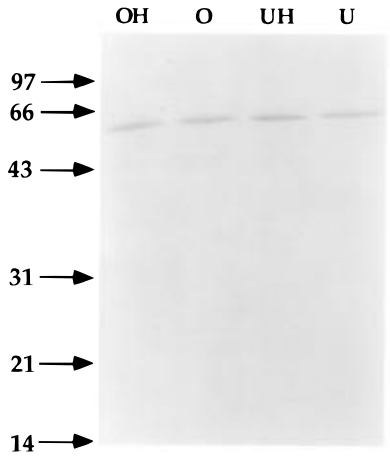

FIGURE 4: Immunoblot of four forms of the rat uterine ER. Equal amounts of cytosol were added to each lane following the appropriate treatment described in Materials and Methods. The percentage of $[^3H]E_2$ binding activity retained by each ER form is shown in parentheses. Estradiol occupied heated (OH, 100%), estradiol occupied nonheated (O, 95%), unoccupied heated (UH, 34%), and unoccupied nonheated (U, 89%). The molecular mass standards were from Bio-Rad and included rabbit muscle phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), hen egg white ovalbumin (43 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and hen egg white lysozyme (14 kDa).

There are several physical alterations in the ER that could define the T to B conversion, including proteolysis of ER, loss of hsp90, or covalent modification of the ER. We used western blot assays to study the possibility that the B population could represent a proteolytic fragment of the full-length ER induced by E_2 binding or heating ER. An immunoblot of four different forms of the ER is shown in Figure 4. The full-length ER migrates at about 65 kDa as expected and treatment of the cytosol with $E_2 \pm$ heating did not significantly decrease the amount of full-length ER and did not lead to the appearance of any new fragments. This antibody has previously been used to map ER proteolysis (Fritsch et al., 1993) and therefore is capable of detecting degradation products of the ER. We have also previously published the observation that heating in vitro does not alter the amount of full-length ER recovered (Fritsch et al., 1992a). We conclude that the partitioning difference between the T and B populations is not due to a difference in the size of the ER protein (i.e., both T and B populations are full-length ER).

One of the possible differences between T and B ER populations could be a difference in the oligomeric state of each. One of the most studied physicochemical changes in the ER has been the transition from an hsp90 bound ER (8S on sucrose gradients) to an hsp90-free ER (5S on sucrose gradients), which corresponds with the transition from a non-DNA-binding ER population to a DNA-binding ER population in vitro (Grody et al., 1982; Carson-Jurica et al., 1990). The 8S complex represents the receptor in an oligomeric complex with hsp90s and other proteins (Pratt, 1990; Kost et al., 1989; Smith et al., 1992; Segnitz & Gehring, 1995). The addition of estrogen with or without heating leads to the dissociation of several members of this complex (especially hsp90) with a resulting 4−5S peak. The 4S peak, which occurs in the absence of heating and is facilitated by high salt concentrations, is thought to represent ER monomer (4S), whereas the 5S peak occurs upon heating or prolonged

Table 1: Partitioning Behavior of the ER in Cytosols Prepared with or without 20 mM Sodium Molybdate[a]

	K_{obs}	
	(−) molybdate	(+) molybdate
U	**2.25** ± 0.03	**2.85** ± 0.08
EO	**2.13** ± 0.18	**2.70** ± 0.04
UH	**0.85** ± 0.31	**2.52** ± 0.11
EOH	**0.71** ± 0.05	**1.54** ± 0.02

[a] Cytosols were prepared in the absence (−) or presence (+) of 20 mM sodium molybdate, then treated with and without [^{3}H]E_2 and heating. The various ER forms were then partitioned in the usual ATPP system plus 20 mM sodium molybdate for both the (+) and (−) molybdate cytosols.

high salt treatment and is thought to represent an ER dimer (possibly a homodimer). The transition from 8S to 5S is called transformation. The kinetics of the 8S to 4−5S transition has been shown to be much slower for the OHT-occupied form of the ER than for the E_2-occupied form (Jordan & Murphy, 1990; Nelson et al., 1988). This difference in the oligomeric state of the ER would be consistent with the data described in Figures 2 and 3. We therefore tested the hypothesis that the T population represented the 8S ER complex and the B population represented the 4−5S ER.

The initial experiment to investigate whether the T to B transition was the same as the 8S to 4−5S transition was to add molybdate to our cytosol prior to adding ligand or heating. Molybdate has been shown in numerous studies to inhibit the loss of hsp90 from the 8S complex and thereby block the transition from 8S to 4−5S (Grody et al., 1982). If our two populations represent 8S and 4−5S, then adding molybdate should effectively block the conversion of the T to B population of ER and the single tube K_{obs} values should be the same for all ER forms. Cytosols were prepared with or without 20 mM sodium molybdate. These cytosols were treated with and without E_2 and with and without heating to 30 °C for 45 min. The cytosols were then partitioned in the same ATPP system as described in Materials and Methods except 20 mM sodium molybdate was present in the ATPP system for both the (+) and (−) molybdate cytosols. Molybdate was added to all of the phase systems to control for any changes in the K_{obs} values induced by the (+) molybdate cytosols because different salts can alter the K_{obs} values of a protein (Walter et al., 1985). For the (−) molybdate cytosol, ligand addition and heating were performed before adding to the ATPP system containing molybdate, whereas for the (+) molybdate cytosol, molybdate was present prior to ligand addition or heating. The presence of molybdate in the phase system does slightly alter the K_{obs} values for the E_2-occupied (EO) and E_2-occupied heated (EOH) ER forms in the control tubes containing (−) molybdate cytosol [see Table 1, (−) molybdate as compared with the K_{obs} values in Figure 3].

Table 1 shows the single-tube partitioning values for four forms of the cytosolic ER with or without molybdate. The differences in the single-tube K_{obs} values for the various ER forms are due only to differing amounts of the T and B ER populations present following ligand and/or heating (Figure 3). Thus, we used a single tube partitioning assay to measure the inhibition of the T to B transition. Molybdate added to the cytosol prior to heating led to a dramatic increase in the K_{obs} value for the UH form compared to the (−) molybdate control value. Molybdate addition to the cytosol also led to an increase in the K_{obs} value of the heated E_2-occupied ER [(+) molybdate EOH] compared with the (−) molybdate EOH control K_{obs} value, but to a lesser extent than for the UH K_{obs} value. This is consistent with a prior study using molybdate, which found that molybdate may not completely prevent the 8S to 5S transition on heating the occupied receptor (Renoir et al., 1990). Therefore, these data are consistent with the two populations representing the 8S (T) and 4−5S (B) ERs. However, molybdate could also be preventing some other change in the ER that is responsible for the T to B transition.

To directly test whether the T population of ER was in the 8S state and the B population was in the 4−5S state we used sucrose density gradient analysis of the T population for two different ER forms. The B population could not be placed directly on 7%−20% sucrose gradients because the dextran from the lower phase was too dense and the sample sank to the bottom of the gradient. If the only difference between the T and B populations was that T was 8S and B was 4−5S, then the T population from every form of the cytosolic ER (U, UH, EO, EOH) should elute as an 8S peak on a sucrose gradient. The T population from two different ER forms was separated from the B population by CCD and placed directly on 7−20% sucrose step gradients. Figure 5A shows the T population from the E_2-occupied nonheated ER (EO) at 4 °C as an 8S peak, and Figure 5B shows the T population from the unoccupied heated ER (UH) as a 4.6S peak. Clearly, the T populations from the EO and UH ER forms are in very different oligomeric states with regard to hsp90. We cannot say with certainty whether the T population of the unoccupied heated ER is in a dimeric state, which should be 5S or a monomeric state at 4S, because the gradients were not sensitive enough to distinguish these two possibilities. Therefore, we conclude that the two ER populations, T and B, do not represent the classic 8S and 4−5S ERs because the T population for the E_2-occupied ER was 8S and the T population for the unoccupied heated ER was 4.6S.

We investigated whether one population of ER might have a different equilibrium dissociation constant (K_d) for [^{3}H]E_2 binding than the other population. Two populations of the ER have previously been described that differ significantly in their K_d values for [^{3}H]E_2 binding (Raymoure et al., 1985, 1986; McNaught et al., 1986, 1990; McNaught & Smith, 1986; Dayani et al., 1990; Nag et al., 1990). To study this we employed equilibrium saturation binding analysis for the T and B populations of the unoccupied and unoccupied heated cytosolic ERs. The T and B populations were separated by two- or three-tube CCD and then incubated at 4 °C with increasing concentrations of [^{3}H]E_2 ± 100-fold molar excess of unlabeled DES. To avoid the problem of each population being in a different environment, mostly PEG for T and mostly dextran for B, each phase was diluted with a corresponding amount of the opposite blank upper or lower phase. Therefore, all tubes were incubated with ligand under the same solution concentrations of PEG, dextran, salts, and buffer. The average K_d values of three independent determinations for each ER population were 0.413 ± 0.059 nM for the unoccupied ER top population (UT), 0.569 ± 0.069 nM for the unoccupied ER bottom population (UB), 0.549 ± 0.031 nM for the unoccupied heated ER top population (UHT), and 1.21 ± 0.09 nM for the unoccupied

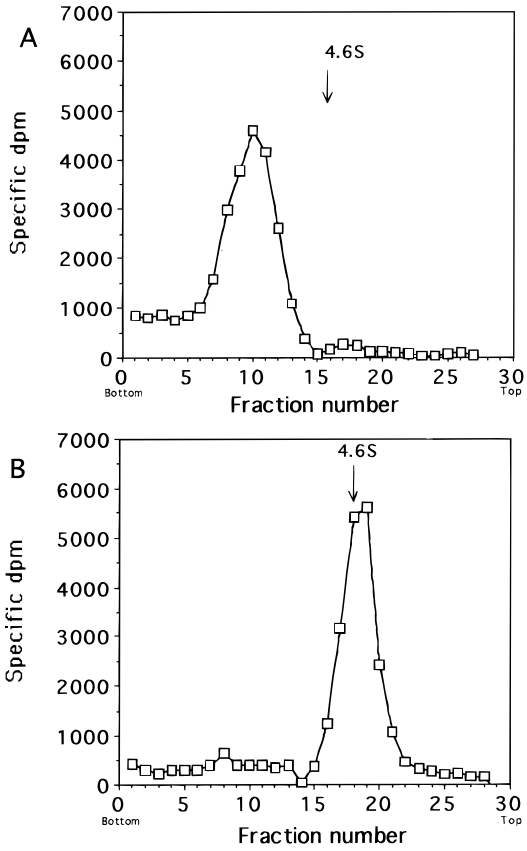

FIGURE 5: Sucrose density gradient analysis of the T populations of the [^{3}H]E_2 occupied nonheated (A) and the unoccupied heated (B) ERs. (A) Cytosol was occupied with [^{3}H]E_2 for 2 h at 4 °C and partitioned, and an aliquot of the upper phase from CCD2 (T population) was placed on a 7%−20% sucrose step gradient in TE buffer and centrifuged overnight. Fractions were collected, and the amount of specific [^{3}H]E_2 binding activity was determined by HAP assay. Dansylated albumin (4.6S) was used as an internal fluorescent marker. (B) Unoccupied heated cytosol was partitioned and an aliquot of the upper phase was placed on a 7%−20% sucrose step gradient in TE and 20 mM sodium molybdate and centrifuged overnight. Fractions were collected and incubated at 10 nM [^{3}H]-E_2 ± 100-fold molar excess of DES for 2 h at 4 °C followed by a HAP assay.

heated ER bottom population (UHB). The K_d values for the UT, UB, and UHT ER populations are all the same value within experimental errors. UHB is significantly different from the other three K_d values ($p < 0.01$) as determined using the Scheffe comparison of multiple means. Although there is a 2-fold lower binding affinity for [^{3}H]E_2 by the B population of the unoccupied heated ER (UHB) compared to the T population (UHT), this effect was not seen in the two populations for the unoccupied nonheated ER (UT, UB). Therefore, this does not represent a characteristic distinguishing the T population from the B population.

We next chose to determine whether there was any difference in the surface hydrophobicity between the two populations. Our laboratory previously showed that upon binding [^{3}H]E_2, the ER undergoes a dramatic decrease in surface hydrophobicity as assayed by affinity partitioning using PEG−palmitate (Hansen & Gorski, 1986; Fritsch et al., 1992b). This change in surface hydrophobicity was localized to the SBD of the ER and was independent of any in vitro heating effects.

Table 2 shows the results from affinity partitioning with PEG−palmitate of three forms of the ER (U, UH, EO).

Table 2: The Hydrophobic Surface Property of the ER in Each ATPP Tube Was Determined Using Affinity Partitioning into PEG−Palmitate[a]

	Δ log K_{obs}		
	unoccupied	unoccupied heated	estradiol occupied
single tube	**0.61** ± 0.07	**0.28** ± 0.03	**0.14** ± 0.01
CCD1	**−0.30** ± 0.04	**−0.31** ± 0.10	**−0.25** ± 0.02
CCD2	**0.55** ± 0.08	**0.67** ± 0.09	**−0.01** ± 0.00

[a] The K_{obs} values for each ER form (U, UH, EO) were determined in the absence and presence of 13 μM PEG−palmitate. The log K_{obs} value in the absence of PEG−palmitate was subtracted from the log K_{obs} value in the presence of PEG−palmitate to generate the Δlog K_{obs} value shown. Δlog K_{obs} was determined for single-tube partitioning and for two-tube CCD (CCD1 and CCD2) for each ER form. A positive value of Δlog K_{obs} demonstrates an increased affinity of the ER for the PEG−palmitate rich upper phase.

PEG−palmitate partitions predominantly into the upper phase, leading to a more hydrophobic environment relative to the phase system with PEG only (Walter et al., 1985; Hansen & Gorski, 1986; Shanbhag & Jonansson, 1974, 1979; Shanbhag & Axelsson, 1975; Axelsson & Shanbhag, 1976; Axelsson, 1978; Johansson & Shanbhag, 1984). The single tube and CCD partition coefficients were obtained in the presence and absence of 13 μM PEG−palmitate. The logarithm of each K_{obs} value was taken and the log K_{obs} in the absence of PEG−palmitate was subtracted from the log K_{obs} value in the presence of PEG−palmitate. Therefore, a positive value of Δlog K_{obs} suggests an increased affinity of the ER for the palmitate rich upper phase, whereas a neutral or negative value suggests no increased affinity for the PEG−palmitate. We interpret an increased affinity of the ER for the PEG−palmitate rich upper phase as due to the presence of hydrophobic surface residues on the protein. For the single-tube partitionings, the unoccupied nonheated ER has hydrophobic surface properties as demonstrated by a relatively large positive Δlog K_{obs} value (0.61). Upon binding E_2, most of the surface hydrophobicity was lost as demonstrated by a small Δlog K_{obs} value for the E_2-occupied ER as previously reported (Hansen & Gorski, 1986; Fritsch et al., 1992b). The unoccupied heated ER showed about a 2-fold increase in K_{obs} when partitioned into PEG−palmitate as demonstrated by a small positive Δlog K_{obs} value. We next performed a two-tube CCD for each ER form in the absence of PEG−palmitate and demonstrated the presence of the two populations of the ER, T (K_{obs} of about 3.5) and B (K_{obs} of about 0.03), as previously shown in Figure 3. We then made the two tubes of the CCD 13 μM in PEG−palmitate prior to the final partitioning for all three ER forms. The B population of ER (found in CCD1), independent of ligand or heating, possessed little or no hydrophobic surface properties, as demonstrated by the Δlog K_{obs} values being negative for the various ER forms. The unoccupied T population, independent of heating, possessed hydrophobic surface properties that were lost upon binding ligand as previously reported (Fritsch et al., 1992b). Thus, there are differences in the hydrophobic surface properties of the two populations as determined by affinity partitioning in PEG−palmitate. The unoccupied T population (found in CCD2), independent of heating, remains capable of responding to estrogen binding with a loss of surface hydrophobicity, whereas the unoccupied B population undergoes no apparent change in its surface hydrophobicity upon binding ligand. The hydrophobic surface properties of the unoccupied ER

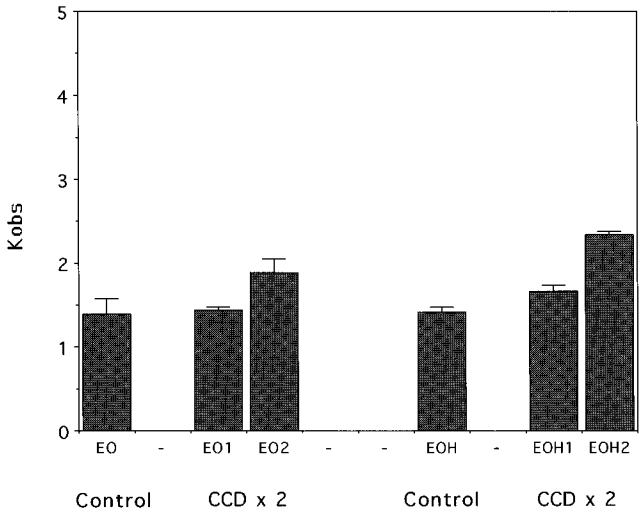

FIGURE 6: CCD of the human ER SBD overproduced in *E. coli*. The SBD from the human ER was overproduced in *E. coli* (Ahrens et al., 1992; Fritsch et al., 1992b). Two forms of the SBD preparation, E_2-occupied nonheated (EO) or heated (EOH), were partitioned using single-tube partitioning (controls) or two-tube CCD (EO1, EO2 and EOH1, EOH2).

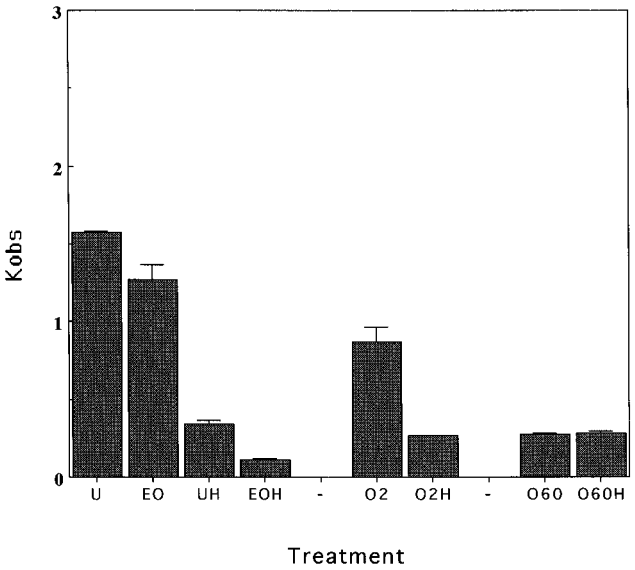

FIGURE 7: Partitioning behavior from MCF-7 whole cell extracts of unoccupied ER and in situ occupied ER forms. Cells containing unoccupied ER were scraped into TEM, homogenized, made 0.4 M in potassium phosphate for 30 min at 4 °C, diluted to 0.2 M salt, and spun in the ultracentrifuge at 4 °C. This whole cell extract was then treated as previously described for rat uterine cytosolic ER in vitro to prepare the four forms of ER: U, EO, UH, EOH. Aliquots of these different ER forms were partitioned in the usual ATPP system. To prepare the in situ occupied whole cell extracts, cells were incubated with 8 nM [^{3}H]E_2 with or without unlabeled DES for 2 min at room temperature or 37 °C followed by 58 min at 4 °C (O2) or 60 min at 37 °C (O60), scraped into TEM, and treated exactly as the unoccupied ER. These whole cell extracts were either maintained at 4 °C (O2, O60) or heated in vitro to 30 °C for 45 min (O2H, O60H) and then partitioned. The fmol of ER/mg of protein in each extract was similar (U, 459; O2, 429; O60, 400). Studies showed that the ER in the O2 extracts was completely occupied prior to scraping the cells (data not shown).

are localized to the SBD (Fritsch et al., 1992b), and the results from Table 2 would suggest a possible difference in the SBD between the T and B populations. However, the amino terminal half of the ER could also be affecting the partitioning behavior of the intact ER protein in the presence and absence of PEG−palmitate. These alterations in surface hydrophobicity had little effect on the ability of the ER to bind [^{3}H]E_2, as demonstrated above by the similar K_d values for E_2 binding by the T and B populations.

To determine whether the T to B transition was localized in the SBD we utilized the SBD of the human ER overproduced in *E. coli* (Ahrens et al., 1992). This SBD construct is missing the amino terminus and almost all of the DNA binding domain. Figure 6 shows that the single-tube K_{obs} values for the E_2-occupied and E_2-occupied heated SBD preparations were identical at 1.4. Two-tube CCD of each of these SBD forms reveals only a small difference between the K_{obs} values in the two tubes. The K_{obs} values range from 1.4 to 2.3. Thus, the overproduced ER SBD preparation behaves more like a single population rather than the full-length receptor that partitions as two populations, as determined by CCD in this ATPP system.

The SBD was also generated by limited trypsin treatment of the rat uterine cytosolic ER. We have previously reported that the single-tube K_{obs} values in this ATPP system for the various ER forms (U, UH, EO, EOH, OHT) after trypsin treatment were identical at 1.5 (Fritsch et al., 1992b). Upon three-tube CCD of the UH and EOH trypsinized ER forms, the K_{obs} values ranged from 0.7 (CCD1) to 2.0 (CCD3) for the three tubes (data not shown). These values are much different than the values of 0.03−3.5 reported for the two populations observed for the intact ER, suggesting that the characteristic defining the two populations is lost upon trypsin treatment. The trypsin-generated SBD, while not completely homogeneous, partitioned more like a single population of ER with an average K_{obs} of 1.5 than the T and B populations of the full-length ER. These data together with the data from the overproduced SBD suggest that the isolated SBD of the ER is not responsible for the T and B populations. Therefore the T and B populations could be due to ligand- and heat-induced changes in the amino terminal and or DNA-binding domains of the ER. Alternatively, the T and B populations could represent different oligomeric states of the ER with repect to protein:protein interactions; however, this protein:protein interaction does not involve hsp90.

ER from Human Breast Cancer Cells (MCF-7) Partition the Same as the Rat Uterine ER. Unoccupied ER from MCF-7 cells were prepared in a whole cell salt extract as described in Materials and Methods. The 0.4 M K_2HPO_4 extraction medium extracts completely both estrogen-occupied and unoccupied forms of ER. We utilized single-tube partitioning because differences in the K_{obs} values in a single-tube partitioning system actually reflect differences in the amount of T and B populations present (Figure 3). The unoccupied ER was salt extracted from MCF-7 cells, and Figure 7 shows the K_{obs} values following four different in vitro treatments [unoccupied nonheated (U), E_2 occupied nonheated (EO), unoccupied heated (UH), and E_2 occupied heated (EOH)]. Although the absolute K_{obs} values are slightly different than for rat uterine cytosolic ER (Figure 3), the overall pattern is the same for the various ER forms. Both ligand binding and heating the MCF-7 extract led to decreases in the K_{obs} value relative to the K_{obs} value for the unoccupied nonheated ER form.

The ER was then occupied in situ by incubating the cells at 37 °C with [^{3}H]E_2 for 60 min (O60), a whole cell extract made by the same method as for the unoccupied ER, and

the extract was partitioned. The K_{obs} value of this in situ occupied ER was about 0.3, which is similar to the K_{obs} value for the MCF-7 in vitro occupied heated ER (EOH). Upon heating the in situ occupied ER extract to 30 °C for 45 min in vitro (O60H), there was no change in the K_{obs} value. This suggests that most of the ER occupied in situ represents ER in the B population. When [^{3}H]E_2 was added to cells for 2 min at room temperature (or 37 °C, data not shown) followed by placing the cells on ice for 58 min, a whole cell extract made by the same method as above, and this extract then patitioned, the ER had a K_{obs} value of 0.85 (O2). The ER was shown to be fully occupied with [^{3}H]E_2 prior to homogenization under these conditions (data not shown). This was intermediate between the K_{obs} value for the ER occupied in vitro (EO) and the K_{obs} value for the ER occupied within the cell for 60 min at 37 °C (O60). When the O2 ER form was heated to 30 °C for 45 min in vitro, the K_{obs} value decreased to 0.3 (O2H). If the data are viewed with regard to the T and B populations, these observations suggest a time dependent change in the ER occurring within the cell upon binding [^{3}H]E_2 at room temperature or 37 °C. This change involves the conversion from the T population to the B population and seems to be temperature dependent in situ as well as in vitro. Thus, the ER from the human MCF-7 cell line behaves similar to the rat uterine ER in vitro. Also, the T to B transition appears to be an event that occurs within the cell after binding estrogen.

DISCUSSION

We demonstrate in this paper that there are at least two populations of ER present in rat uterine cytosol that can be distinguished by their K_{obs} values in an ATPP system. The two populations have been designated T, which represents a population of ER that partitions preferentially into the top phase with a K_{obs} value between 3 and 6, and B, which represents a population of ER that partitions preferentially into the bottom phase with a K_{obs} value of 0.01−0.03. Countercurrent distribution in an ATPP system can be used to rapidly and efficiently separate these two populations. Most of the unoccupied ER extracted into a hypotonic cytosol exists in the T population and the addition of E_2 or heating the cytosol in vitro leads to the conversion of the T population to the B population. The physicochemical difference between the T and B populations has been partially characterized in this work. The T to B transition is significantly less when an antiestrogen is the ligand (Figure 3). The difference between T and B is not due to proteolysis of the ER (Figure 4). The T and B populations of ER have the same estrogen binding affinity at 4 °C. Although molybdate can significantly inhibit the estrogen- and heat-induced T to B transition in vitro (Table 1), the T to B transition is not simply due to the loss of hsp90 or conversion of the ER from an 8S to a 4−5S complex (Figure 5).

The exact location within the ER protein that dictates the difference between the T and B populations remains to be determined. The loss of the two populations using the *E. coli*-overproduced SBD and the trypsin-generated SBD suggests one of two possibilities. First, and our favored view, is that the difference distinguishing the T from the B population of ER could be localized within the amino terminal portion of the ER. Thus, removal of the A/B and DNA binding domains results in the loss of the T to B transition. Since this transition is ligand dependent (Figure 3), it would suggest that ligand binding can allosterically effect the amino terminal conformation or structure of the ER. This is consistent with previous observations from our laboratory showing that DNA binding can allosterically alter the SBD of the ER (Fritsch et al., 1992a).

Second, the difference between the T and B populations of ER could be localized within the SBD. In this study, a significant difference in the surface hydrophobicity between the two populations of ER for the unoccupied ER forms (Table 2) was observed. The T population behaved similar to the isolated SBD (Fritsch et al., 1992b) in the PEG−palmitate phase system. The unoccupied T population moved dramatically into the PEG−palmitate rich upper phase, and upon the addition of E_2 these hydrophobic surface properties were lost. These effects were independent of heating the T population as was previously demonstrated for the SBD (Fritsch et al., 1992b). However, the B population showed no hydrophobic surface properties in the presence or absence of ligand. One possible conclusion from these data is that there is a difference between the two populations localized within the SBD. However, changes in the ER localized to the amino terminal or DNA binding domains cannot be excluded.

The oligomeric state of the ER has been shown by our laboratory (Fritsch et al., 1993) and others (Golding & Korach, 1988; Nelson et al., 1989) to be dependent upon ligand binding and heating, consistent with the T to B transition. The SBD could be the site regulating the T to B transition by its interaction with other proteins or itself (the monomer to dimer transition). Some other protein interacting with the ER SBD could play a key role in dictating the partitioning behavior of the ER. The *E. coli*-overproduced SBD preparation may lack this protein, and the trypsin-generated SBD may result in proteolysis of this protein, both resulting in a relatively homogeneous single population of estrogen binding activity. We have shown above that hsp90 dissociation is not involved in the T to B transition. The ER:hsp90 interaction sites have been mapped to the SBD (Dalman et al., 1991; Denis et al., 1988; Howard et al., 1990; Pratt et al., 1988; Chambraud et al., 1990; Guiochon-Mantel et al., 1989; Sabbah et al., 1989). These data support the hypothesis that the region of the ER dictating the T to B transition is not necessarily the SBD. However, several other proteins have been demonstrated to be complexed with the ER, especially within a cytosolic mixture (Segnitz & Gehring, 1995; Johnson & Toft, 1994).

The T to B transition could also represent the monomer to dimer transition. ER dimerization has recently been shown to be ligand dependent in vivo (Wang et al., 1995). The amino acid residues that mediate dimerization have been proposed to be located within the SBD and possibly within the DNA binding domain (Luisi et al., 1991). Preliminary data from our laboratory (data not shown) indicate that the Top and Bottom populations of the E_2-occupied ER both elute as similar large molecular weight complexes by gel filtration, arguing that the T to B transition is more complicated than a simple monomer to dimer transition. Even if one population of the ER were shown to be monomer and the other a dimer, as determined by size, there remains controversy as to whether the dimer is a homodimer or a heterodimer (Furlow et al., 1993; Murdoch et al., 1995). Whether the SBD overproduced in *E. coli* or generated by trypsin treatment are in a monomeric, dimeric (homodimer

or heterodimer), or mixed state is also not known. However, we have previously shown that the overproduced SBD exists as a mixture of monomer and higher M_r complex (greater than 152 kDa) for both the unoccupied and E2 occupied forms (Fritsch et al., 1992b). Again, this shows that a clearly heterogeneous (as determined by size) population of ER−SBD partitions as a homogeneous protein population (Figure 6). This, along with the fact that the T population of ER can exist as both 8S and 4−5S argues strongly against size alone dictating partitioning behavior and that other surface properties of the ER are important for distinguishing T and B populations. Other proteins interacting with the amino terminal half of the ER could also account for the T to B transition. Further studies to definitively identify the exact region of the ER responsible for the T to B transition are necessary.

Finally, changes in the surface charge of the ER or the ER:protein complex could alter its partitioning behavior. A very large difference in the surface charge between the nontransformed glucocorticoid receptor and the heat-transformed glucocorticoid receptor has been reported using ATPP (Andreasen, 1982). The surface charge of the ER can be changed by numerous factors such as binding to nucleic acids, oligomerizing with other highly charged proteins, or direct enzymatic alteration of the receptor's surface charge by phosphorylation or dephosphorylation (LeGoff et al., 1994; Arnold et al., 1995). Thus, T and B could represent populations of the ER modified by one or all of the above processes.

Although the exact physicochemical alteration in the ER responsible for the T to B transition is not known, it appears to be functionally important as the transition occurs within the intact cell. We have demonstrated that the single tube partitioning behavior of the ER from the human MCF-7 breast cancer cell line is similar to the rat uterine cytosolic ER, suggesting the presence of two populations of ER in MCF-7 extracts. The data from Figure 7 suggest that the transition that occurs upon heating the E_2-occupied ER in vitro may represent a similar change that occurs to the ER within the cell upon binding E_2. Since we have characterized this transition for the rat uterine ER as a conversion of the T population to the B population, we conclude that the T to B transition is occurring within the cell upon the addition of E_2. This process appears to be ligand, temperature, and time dependent but independent of any interaction with hsp90 and may represent a functionally important change in the ER necessary for transcriptional activation of estrogen dependent genes.

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