Conformational and Electrostatic Properties of Unoccupied and Liganded Estrogen Receptors Determined by Aqueous Two-Phase Partitioning†

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ABSTRACT: The technique of aqueous two-phase partitioning (ATPP) has been used to characterize conformational and electrostatic properties of unoccupied and liganded rat uterine estrogen receptors. The adaptation of the hydroxylapatite receptor assay with ATPP systems has permitted estrogen receptor (ER) partition coefficients to be accurately determined, even when the partitioning process results in significant loss of ER binding capacity. The pH and salt dependences of estrogen receptor partition coefficients indicate that the theory governing partitioning behavior can be accurately applied to partitioning data obtained with crude cytosols. This technique has revealed a ligand-induced change in the properties of the unoccupied receptor that precedes the process of heat-induced transformation in vitro. The difference in partitioning behavior between unoccupied and nontransformed estrogen receptor is observed in all combinations of buffers and salts tested and is of equal magnitude as the difference between partition coefficients of nontransformed and transformed ER. The partition coefficients of both unoccupied and nontransformed ER are constant over the ER concentration range in which binding cooperativity has been previously demonstrated. The combined effects of salt and pH on ER partition coefficients indicate a p*I* of ~5.5 for both unoccupied and nontransformed estrogen receptors. However, the partition coefficients at the p*I* differ. It is concluded that estradiol binding to its unoccupied receptor results in a change in surface properties of the ER monomer that is independent of receptor transformation and makes the receptor less hydrophobic.

Largely due to technical limitations, the physical properties and interactions of unoccupied steroid receptors remain essentially unknown (Sherman & Stevens, 1984). Although there has been previous evidence that susceptibility to thermal and photodegradation of unoccupied estrogen receptor (ER)[1] and nontransformed ER differs (Katzenellenbogen et al., 1973, 1975), the relationship between these ER forms remains obscure.[2] Since nontransformed ER has low affinity for DNA and traditionally has been thought to reside in the cytoplasm, it has been easy to assume that there is little difference between unoccupied and nontransformed steroid receptors and to refer to the nontransformed receptor as the "native" ER form (Molinari et al., 1977). However, recent studies [Welshons et al., 1984; King & Greene, 1984; see review by Gorski et al. (1984)] have demonstrated a nuclear, unoccupied estrogen receptor and emphasize a renewed need to investigate directly the behavior and characteristics of unoccupied steroid receptors. As a result, we have adopted a technique that allows one to assess quantitatively the behavior of both unoccupied and liganded receptors under identical conditions. Because aqueous two-phase partitioning is independently sensitive to both conformational and electrostatic properties of proteins, it can be used to distinguish between ligand-induced changes in ER conformation, molecular charge, and (or) state of aggregation. Furthermore, since phase partitioning is governed by thermodynamic principles, it provides an alternative approach to the more commonly used hydrodynamic techniques that discriminate on the basis of size and shape under nonequilibrium conditions.

Behavior of charged solutes in biphasic systems is described by the electrochemical potential, μ:

$$\mu = \mu^\circ + RT_p \ln \gamma C + ZF\psi \quad (1)$$

At partitioning equilibrium, the electrochemical potential of the solute must be equal in both phases. This yields the equation for the standard free energy of transfer in terms of the equilibrium distribution of solute and the electrical potential difference ($\Delta\psi$) across the phase boundary:

$$\Delta G^\circ_{tr} = \mu^\circ_{top} - \mu^\circ_{bottom} = RT_p \ln \frac{(\gamma C)_{top}}{(\gamma C)_{bottom}} + ZF\Delta\psi \quad (2)$$

Experimentally, one determines the solute concentration in each phase and expresses them as the partition coefficient, K_{obsd}:

$$K_{obsd} = \frac{\text{measured concn in top phase}}{\text{measured concn in bottom phase}} \quad (3)$$

From eq 2 and 3, neglecting activity coefficients, one arrives

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[1] Abbreviations: ER, estrogen receptor(s); E_2, 17β-estradiol; ATPP, aqueous two-phase partitioning; PEG, poly(ethylene glycol); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; TED, 10 mM Tris-HCl, 1.5 mM EDTA, and 0.5 mM dithiothreitol; HED, 10 mM Hepes, 1.5 mM EDTA, and 0.5 mM dithiothreitol; PED, 10 mM potassium phosphate, 1.5 mM EDTA, and 0.5 mM dithiothreitol; μ°, standard-state chemical potential; γ, activity coefficient; C, solute concentration; R, gas constant; T_p, absolute temperature; F, Faraday constant; Z, solute charge; ψ, solvent electrical potential; K_{obsd} (K_{obs} in figures), partition coefficient; HAP, hydroxylapatite; NSB, nonspecific binding.

[2] Nontransformed ER refers to estrogen–receptor complexes formed in vitro by incubating unoccupied ER with ligand at 0 °C. Heat, salt, or ATP treatment of this ER form induces physicochemical changes in receptor properties that have collectively been termed transformation and/or activation.

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at the fundamental equation governing equilibrium partitioning behavior (Albertsson, 1971):

$$\ln K_{obsd} = \ln K_0 + \frac{ZF}{RT_p}\Delta\psi \quad (4)$$

where K_0 is the ratio of solute concentrations when either molecular charge or interfacial potential is zero. Thus, the partition coefficient is a direct measure of the standard free energy of transfer and is independently influenced by solute–solvent interactions (i.e., K_0) and electrostatic properties (i.e., Z) of the molecule being partitioned.

In aqueous two-phase partitioning, the phases are formed from mixtures of water-soluble, high molecular weight polymers such as dextran and poly(ethylene glycol). Unlike most biphasic systems, ATPP systems are highly aqueous throughout and provide a more subtle difference in solvent properties of the phases. As a result, the partition coefficients of most biomolecules in ATPP systems are much closer to unity and can be used as a probe of the solute's molecular properties. Furthermore, the phase environments consisting of 10–20% polymers and salts create solvent conditions that are much closer to those found in cells (Fulton, 1982) than most other techniques used for physicochemical analysis.

The partition of proteins, nucleic acids, organelles, and whole cells in aqueous two-phase partitioning systems has been elegantly presented [Albertsson, 1971; see Albertsson (1978) for a review]. Previous studies by Andreasen have used ATPP to study a number of characteristics of the transformation process of the rat liver glucocorticoid receptor (Andreasen, 1978, 1981, 1982; Andreasen & Mainwaring, 1980) and to characterize aberrant receptor properties in glucocorticoid receptors isolated from cultured mouse lymphoma cells (Andreasen & Gehring, 1981). This report describes the first application of ATPP in combination with the hydroxylapatite (HAP) ER assay to the study of rat uterine estrogen receptors. Particular emphasis is given to the properties of unoccupied estrogen receptors and how they differ from those of the liganded forms of the molecule.

Materials and Methods

Dextran (M_r 461 000 and 510 000), poly(ethylene glycol) (M_r 8000), leupeptin, and aprotinin were purchased from Sigma Chemical Co. 17β-[2,4,6,7-^{3}H]Estradiol (93–104 Ci/mmol) was purchased from New England Nuclear and Amersham. Immature female rats between the ages of 19 and 21 days were obtained from Holtzman (Madison, WI). Bio-Gel HT hydroxylapatite (HAP) was purchased from Bio-Rad Laboratories. All other chemicals were of reagent grade. All procedures were performed at 0–4 °C except where indicated.

Preparation of ER. Uteri were dissected, stripped of fat, and immediately placed in buffer containing 1.5 mM EDTA, 0.5 mM dithiothreitol, and either 10 mM Tris-HCl (TED), 10 mM Hepes (HED), or 10 mM potassium phosphate (PED). In some experiments, the buffer also contained 100 ng/mL–100 μg/mL both leupeptin and aprotinin. The uteri were then minced and soaked in buffer for 60 min to remove nonspecific binding contaminants carried over from serum. Minced uteri were homogenized at five to six uteri per milliliter of the above buffers in a chilled glass–glass tissue grinder. Cytosol was prepared by centrifugation at 130000g for 60 min in a Ti50 rotor (Beckman). ER concentration of typical cytosols ranged from 4 to 6 nM.

In experiments with unoccupied estrogen receptor, aliquots of the cytosol were immediately partitioned as described below. In experiments involving liganded receptor, nontransformed ER was formed by incubating cytosol with 10 nM [^{3}H]estradiol for 2.5–3 h at 0 °C. ER transformation was induced by incubation for the last 60 min at 30 °C. In all cases, parallel incubations were performed with 200-fold excess unlabeled estradiol to determine nonspecific binding.

Preparation of Phase Systems. Dextran and poly(ethylene glycol) (PEG) were initially stored as 15–20% (w/w) stock solutions in TED, HED, and PED buffers. Equilibrated undiluted phase systems composed of 9% (w/w) each of dextran and PEG and various concentrations of salts were formed by combining respective stock solutions and buffer and vortexing rapidly. Aliquots (0.9 mL) of this turbid polymer and salt mixture were added to 0.6 mL of buffer and cytosol by using a positive displacement pipet (SMI) and allowed to sit at 0 °C for 2–3 min. This yielded the standard phase systems used throughout these studies of 5.4% (w/w) of each polymer, with different salts and salt concentrations. The final mixtures of polymers, salts, cytosol, and buffer were vortexed for 5–15 s and then centrifuged for 5 min at 600g to form the characteristic biphasic systems. Duplicate unassayed phase systems were prepared and used to determine upper and lower phase H_2O content in each experiment. Phase H_2O was determined by placing a sample of phase on a glass fiber filter and weighing the filter before and after 24 h at 60 °C. The pH of the final phase system was manipulated by adding small amounts of concentrated HCl or NaOH to undiluted phase mixture, prior to combination with cytosol. The partitioning pH was determined by measuring the pH of the remaining phase systems after samples had been removed for HAP assay. Under the conditions described above, the upper phase was 90.5 ± 0.9% (SD, n = 29) H_2O, 63% of the total volume and PEG rich, while the bottom phase was 80.4 ± 0.8% (SD, n = 29) H_2O, 37% of the total volume and dextran rich. These characteristics of the phase systems were independent of buffer, salt type, pH, and molecular weight of the dextran.

HAP Assay of Phases. After the final phase mixtures were vortexed and centrifuged, 0.15–0.20 mL of each phase was removed with a positive displacement pipet (SMI) and placed in a separate tube. If the unoccupied ER had been partitioned, phase samples were incubated with an equal volume of 20 nM [^{3}H]estradiol with or without 4 μM unlabeled estradiol for 2.5–3.0 h before the HAP assay in order to saturate all ER in the phases. Phase samples containing previously formed ER complexes were directly assayed for receptor. HAP assay was performed essentially as described (Williams & Gorski, 1974) with the following minor modifications. Aliquots (0.2 mL) of 70% HAP in 50 mM Tris-HCl, pH 7.4 (25 °C) (T), buffer were added to all phase samples. One milliliter of homogenization buffer was also added to dilute the viscous phases. After a 30-min incubation with several mixings, 1.0 mL of T buffer was added, and the samples were centrifuged at 1000g for 1–2 min. The HAP pellets were washed 4 times by resuspension in 2 mL of T buffer, followed by centrifugation at 1000g for 1–2 min. The final pellet was extracted with 1.0 mL of absolute ethanol at room temperature. Aliquots of 0.5 mL were counted in 3.5 mL of 10% (v/v) Bio-Solv (Beckman), 0.5% (w/v) 2,5-diphenyloxazole, and 0.03% (w/v) 1,4-bis(5-phenyl-2-oxazolyl)benzene in toluene-based scintillation cocktail at 35–39% efficiency.

Calculation of Partition Coefficients and ER Recovery. Each partition coefficient was calculated from two to six partitionings of both total and nonspecific binding (NSB). The cpm of [^{3}H]E_2 were corrected for counting efficiency, normalized to phase H_2O, and expressed as dpm of [^{3}H]E_2 per milliliter of phase H_2O. The mean dpm of [^{3}H]E_2 per milliliter

Table I: Hydroxylapatite Estrogen Receptor Assay in Buffer and Phases

ER form	ER concn[a] in incubn mixture[b]		
	TED	TED/0.1 M Li_2SO_4	
		upper phase	lower phase
unoccupied	1.00 ± 0.03	1.07	1.08 ± 0.01
nontransformed	1.02 ± 0.02	1.10 ± 0.01	1.06
transformed	1.00 ± 0.01	1.07 ± 0.01	1.03 ± 0.02

[a] Each value represents the mean ± standard error of duplicate determinations (except where no standard error is indicated) and is normalized to the unoccupied TED value. [b] Identical aliquots of cytosols containing either unoccupied, nontransformed, or transformed ER were incubated either with TED or with upper and lower phases from TED/0.167 M Li_2SO_4 systems such that final phase environments were identical with those used in actual partitionings as determined by phase H_2O content. Unoccupied ER was incubated for 2 h as indicated above and then saturated with 10 nM [^{3}H]estradiol for 2.5 h prior to HAP assay. Liganded ER was formed as described under Materials and Methods, incubated for 2 h in the various mixtures, and assayed for ER at the same time as the unoccupied ER. The rest of the HAP assay was performed as described under Materials and Methods. The molecular weight of dextran was 461 000.

of phase H_2O values of NSB were subtracted from the mean dpm of [^{3}H]E_2 per milliliter of total binding to give a mean ER dpm of [^{3}H]E_2 per milliliter value in each phase. The partition coefficient, K_{obsd}, was calculated as the ratio of mean ER concentrations in the upper and lower phases (eq 3). The standard error (SE) of each individual K_{obsd} was calculated according to

$$\text{SE of } K_{obsd} = \sqrt{(\%\text{ SE of ER in top})^2 + (\%\text{ SE of ER in bottom})^2}$$

The number of determinations is expressed either as nk where n is the number of tubes per partition coefficient and k is the number of partition coefficients determined under equivalent conditions or, in some cases, as the mean of several K_{obsd} values. In all cases, identical HAP assays were performed on unpartitioned cytosols to determine the initial ER content. ER recovery was calculated by comparing the amount of receptor recovered per phase system after being partitioned with the amount initially added.

Results

HAP Assay of ER in Buffers and Phases. In order to measure valid partition coefficients under partitioning conditions which may result in ER loss, we have adapted the hydroxylapatite ER assay (Williams & Gorski, 1974) for use with ATPP. Table I shows the results of an experiment in which we compared identical HAP assays of unoccupied and liganded ER incubated in TED and TED/0.1 M Li_2SO_4 phase environments. Results indicate that the same amount of receptor is measured by the HAP assay whether ER is incubated in TED buffer, upper phase or lower phase, and whether cytosol is incubated with phase environments before or after incubation with [^{3}H]E_2. Since the length and order of the incubations and the phase environments exactly duplicated those used during actual partitionings, we conclude that the receptor partition coefficients as determined by the HAP assay are valid under these conditions. The results in Table I also indicate that ligand binding and heating for 60 min at 30 °C have no effect on receptor stability per se as measured by the binding capacity of cytosols and are consistent with previous reports that warming of liganded ER does not result in receptor loss (Notides & Nielsen, 1974). In two similar experiments comparing unoccupied and nontransformed ER, we have obtained essentially identical results.

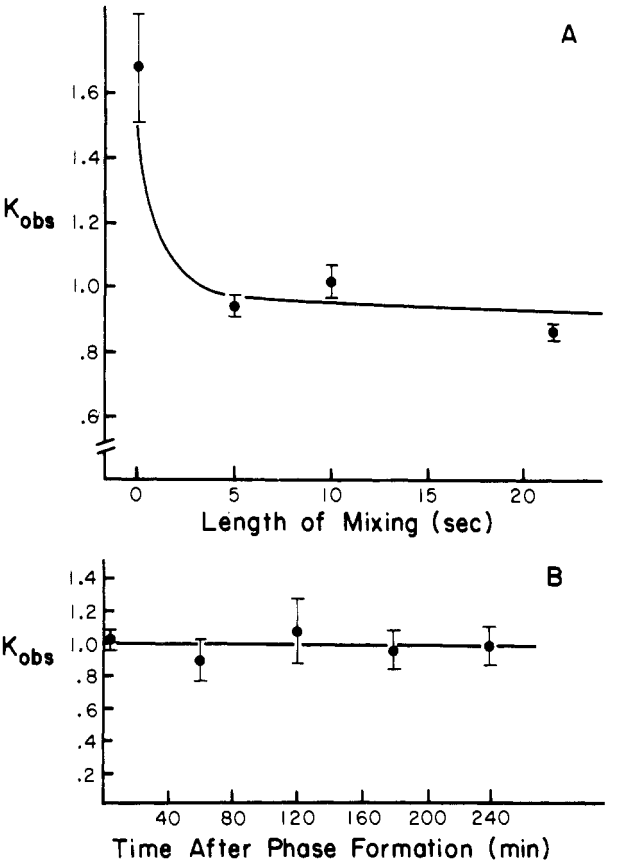

FIGURE 1: Determination (A) and stability (B) of the equilibrium value of the unoccupied estrogen receptor parition coefficient in TED/0.1 M Li_2SO_4 systems. Tubes containing unlabeled rat uterine cytosol, 5.4% (w/w) of both dextran (M_r 461 000) and poly(ethylene glycol), 0.1 M Li_2SO_4, and TED buffer, pH 8.0, were vortexed for the indicated times (A) or for 10 s (B), centrifuged for 5 min at 600g, and placed at 0 °C. Either immediately (A) or at the times indicated (B), aliquots of each phase were removed, assayed as described under Materials and Methods, and used to calculate the corresponding partition coefficients. In (A), the amount of ER recovered at 0, 5, 10, and 20 s of vortexing was 69%, 66%, 68%, and 75%, respectively. In (B), the amount of ER recovered at 1, 2, 3, and 4 h after phase formation was 74%, 74%, 67%, and 73%, respectively. Each data point represents the mean ± the standard error of one K_{obsd} derived from triplicate partitioning (A) or the mean ± standard error of two to six K_{obsd} values determined under similar conditions (B).

Establishment of ER Partitioning Equilibrium. The ability to describe ER partitioning behavior using eq 4 requires that the ER has reached partitioning equilibrium. This in turn depends on the choice and extent of mixing procedures. When the undiluted polymer mixture composed of aliquots of polymer and salt stock solutions is rapidly mixed by stirring, the initially clear mixture becomes turbid and much less viscous. When this solution is combined with cytosol and mixed by gentle vortexing, the same turbidity formation phenomenon is observed. In Figure 1A, the partition coefficient of the unoccupied ER is shown as a function of length of vortexing after turbidity formation in TED/0.1 M Li_2SO_4 phase systems. These data indicate that partitioning equilibrium is reached within 5 s after mixing by vortexing but not at the point of initial turbidity formation (0 s). Another important observation from this experiment was that the amount of receptor recovered after mixing by vortexing was only 70% of the amount added, even at the point of initial turbidity change. Since loss of binding capacity was maximum prior to attainment of equilibrium, we conclude that the formation of a turbid mixture is associated with the loss of ER, but because the loss occurs prior to equilibration, there is no effect on the equi-

Table II: Partition Coefficients of Unoccupied, Nontransformed, and Transformed Estrogen Receptors in Buffers and Salts[a]

	TED[b]					PED[c]		HED[d]		
ER form	TED	0.1 M NaCl	0.05 M K_2SO_4	0.1 M LiCl	0.1 M Li_2SO_4	PED	0.06 M Li_2SO_4	HED	0.1 M NaCl	0.033 M Li_2SO_4
unoccupied	0.03 (2)	0.03 ± 0.01 (2)	0.06 (2)	0.10 (4)	1.00 ± 0.04 (49)	0.28 ± 0.03 (6)	1.84 ± 0.04 (4)	0.06 (2)	0.03 (2)	0 83 ± 0 04 (2)
nontransformed	ND[e]	0.01 (2)	ND	0.05 (4)	0.44 ± 0.03 (19)	ND	1.04 ± 0.03 (4)	ND	<0.01 (2)	0.63 ± 0.02 (2)
heat transformed	ND	ND	ND	0.02 (4)	0.17 ± 0.05 (13)	ND	0.07 ± 0.01 (4)	ND	<0.01 (2)	0.58 ± 0.01 (2)
pH	8.0	8.1	8.2	8.0	8.0	8.0	7.7	8.5	8.4	8.4

[a] Numbers in parentheses indicate *nk*. If the standard error of the mean was less than 0.01, only the mean is indicated. The molecular weight of dextran was 461 000. [b] Partitionings were buffered with 10 mM Tris-HCl, 1.5 mM EDTA, and 0.5 mM dithiothreitol. [c] Partitionings were buffered with 10 mM potassium phosphate, 1.5 mM EDTA, and 0.5 mM dithiothreitol. [d] Partitionings were buffered with 10 mM Hepes, 1.5 mM EDTA, and 0.5 mM dithiothreitol. [e] Not determined.

librium value of the ER partition coefficient. This loss of ER could reflect either receptor partitioned at the interface or ER that was denatured during mixing and is discussed more thoroughly below.

When unoccupied ER was partitioned under conditions identical with those in Figure 1A except that phase samples were removed at various times after centrifugation instead of immediately (Figure 1B), it was found that the partition coefficient of the unoccupied ER was stable for at least 4 h after phase formation and equal to the equilibrium value determined in Figure 1A. Furthermore, the ER recoveries were also equal, indicating that no further receptor loss occurred in either phase during the 4-h incubations at 0 °C following centrifugation. Identical results were obtained with nontransformed and transformed ER through at least 2 and 1 h, respectively (longest times tested, data not shown).

Partition Coefficients of ER in Buffers and Salts. In order to determine if the electrostatic properties of the ER influence partitioning behavior, we initially determined the salt dependences of ER partition coefficients. Salt effects previously have been shown to result from the different partitioning behavior of the anion and cation (Johansson, 1970). This sets up an electrical potential difference across the interface ($\Delta\psi$), the magnitude of which is dependent on the types and concentrations of salts used (Reiterman et al., 1972; Johansson, 1974; Brooks et al., 1984). Thus, if the molecule is charged, eq 4 predicts that the K_{obsd} should be a sensitive function of salt. We determined base-line partitioning values of unoccupied ER in phase systems composed of various buffers and then determined the effects of salts on ER partition coefficients relative to buffer only (Table II). Previously, Albertsson (1971) has shown that the relative abilities of anion and cation to raise the partition coefficient of a number of purified acidic proteins are $Li^+ > Na^+ > K^+$ and $SO_4^{2-} > Cl^-$, respectively. Table II indicates that the salt dependence of the partitioning behavior of all three ER forms follows these previously determined relationships, even though the ER is being partitioned along with other cytosolic components. Similarly, we have observed essentially identical effects of Li_2SO_4 and NaCl relative to Hepes buffer on the K_{obsd} of *Escherichia coli* ^{35}S-labeled RNA polymerase σ factor and holoenzyme purified to homogeneity (data not shown). Therefore, the effects of salts on estrogen receptor partitioning appear to be directly mediated through the combination of receptor charge and interfacial potential.

Examination of Table II also indicates that the combination of ligand and heat produces a number of changes in partitioning behavior of the rat uterine estrogen receptor. Similar conditions to those that lower the partition coefficient of the nontransformed ER (60 min at 30 °C) have previously been shown to induce both slow dissociation kinetics (Weichman & Notides, 1977; Sakai & Gorski, 1984) and a 5S sedimentation coefficient (Shyamala & Gorski, 1969; Notides & Nielsen, 1974) and cause the ER to gain the ability to bind tightly to DNA (Yamamoto, 1974) and various other polyanions. This is consistent with previous findings that steroid receptor transformation can be observed with ATPP (Andreasen, 1978; Andreasen & Mainwaring, 1980). It should be noted, however, that there are differences in the magnitude of the heat-induced change in the ER K_{obsd} in the three buffers. This may reflect differences in the extent of heat transformation in the different buffers or alternatively could be due to one or all of these buffers directly interacting with the transformed state of the ER.

A more unique observation is that the unoccupied estrogen receptor partitions differently than the nontransformed ER in all combinations of buffers and salts tested and at salt concentrations from 0.1 to 0.3 M (Table II). The magnitude of this initial change in unoccupied ER K_{obsd} is similar to that induced by heat transformation and indicates that the molecular differences between unoccupied and nontransformed ER are as dramatic as the differences in properties between nontransformed and transformed ER.

To determine if receptor proteolysis was involved, we compared partition coefficients of unoccupied, nontransformed, and transformed ER from uteri homogenized in the presence of the protease inhibitors leupeptin and aprotinin and subsequently partitioned in TED/0.1 M Li_2SO_4 systems at pH 8.0. The K_{obsd} values of all three ER forms were found to be very similar to the mean values listed in Table II over the inhibitor range from 100 ng/mL to 100 μg/mL (data not shown). Thus, neither the receptor structures nor the transitions caused by ligand and heat are apparently affected by serine and cysteine proteases that may be released into the cytosol upon homogenization.

Recovery of ER in Buffers and Salts. As indicated in Figure 1A,B, the recovery of unoccupied ER in TED/0.1 M Li_2SO_4 systems at pH 8 is only 70%. A complete analysis of salt effects on ER recovery is shown in Table III. These data are derived from the same partitionings as used in Table II and yield a number of interesting observations. Essentially all unoccupied and nontransformed ER are recovered after partitioning in all systems with TED buffer and salt concentrations $\leq$0.1 M. In all buffers, but at ionic strengths from 0.1 to 0.3 M, lithium sulfate dramatically reduces recovery of all forms of the ER. Whereas recovery of both unoccupied and nontransformed ER in Tris-buffered systems is the same, heat-transformed ER recovery is reduced under identical

Table III: Recovery of Unoccupied, Nontransformed, and Transformed Estrogen Receptors in Buffers and Salts[a]

	ER recovery (%)[b]								
	TED				PED		HED		
ER form	TED	0.1 M NaCl	0.1 M LiCl	0.1 M Li_2SO_4	PED	0.06 M Li_2SO_4	HED	0.1 M NaCl	0.033 M Li_2SO_4
unoccupied	114 ± 4	99 ± 22	113 ± 2	64 ± 3	90 ± 9	81 ± 2	69 ± 2	89 ± 3	55 ± 2
nontransformed	ND[c]	107 ± 8	96 ± 9	65 ± 4	ND	67 ± 2	ND	115 ± 22	56 ± 2
transformed	ND	ND	74 ± 12	39 ± 11	ND	42 ± 6	ND	133 ± 6	66 ± 1

[a] These data are derived from the same partitionings as used in Table II. *nk* and the partitioning pH are the same as indicated in Table II. [b] ER recovery is defined as the amount of ER assayed after phase partitioning divided by the amount of ER initially added. [c] Not determined.

conditions. Conversely, in Hepes-buffered systems, much less unoccupied ER is recovered, but ligand binding and heat transformation increase receptor recovery to ≥100%.

As shown previously, Table I and Figure 1A,B indicate that receptor loss at ~pH 8 is caused by the act of mixing. Since ER loss during mixing only occurs in specific salts and at higher salt concentrations, it seems likely that the loss is caused by denaturation of a fraction of the ER during partitioning rather than representing ER partitioned at the interface. Since there is no clear correlation between ER partition coefficients and ER recovery (i.e., differences in partition coefficients are independent of differences in extent of recovery), ER recovery can be thought of as an additional probe of receptor properties which reflects the effect of solution environment on ER stability during mixing.

Effect of Receptor Concentration on Unoccupied and Nontransformed ER Partition Coefficients. Since the estrogen receptor can participate in concentration-dependent subunit–subunit interactions (Notides et al., 1981; Sakai & Gorski, 1984), we determined ER partition coefficients as a function of ER concentrations ranging from 30 pM to 1 nM. In the experiment shown in Figure 2, it was found that the partition coefficient of unoccupied ER does not change over ER concentrations from 0.2 to 0.8 nM. The nontransformed ER partition coefficient is also constant over a range of 0.2–1.0 nM ER. This is the same ER concentration range that the Hill coefficient varies from 1.0 to 1.6 (Notides et al., 1981). In two other overlapping experiments with unoccupied ER covering ER concentrations from 30 pM to 0.5 nM, results were essentially identical (data not shown) and indicated clearly that unoccupied ER partition coefficients are concentration independent over this 30-fold ER concentration range.

"Cross-Point" Analysis of Unoccupied and Nontransformed ER. In order to determine whether the difference in partitioning behavior of the unoccupied and nontransformed ER observed in Table II is due to a difference in molecular charge or a difference in conformation, we performed the cross-point analysis initially described by Albertsson (Albertsson et al., 1970). The principles behind this approach may be seen in eq 4. Briefly, when either the interfacial potential or the net molecular charge is zero, the partition coefficient will only reflect the solvent–solute interactions in each phase (K_0). Different salts have different effects on the magnitude of the interfacial potential but should in theory have little effect on the K_0 value unless their ions bind specifically to the protein. Altering pH changes the net charge on the molecule. Therefore, the profiles of K_{obsd} vs. pH determined in the presence of different salts should intersect (the cross-point) at the pH at which the net charge on the molecule is zero. The pH at the point of intersection should be the isoelectric point of the molecule, and the K_{obsd} at the point of intersection should be the K_0. Albertsson has used this method to establish an excellent correlation between the p*I* of over 15 different proteins as determined by the cross-point analysis and the more commonly used technique of isoelectric focusing (Albertsson et al., 1970).

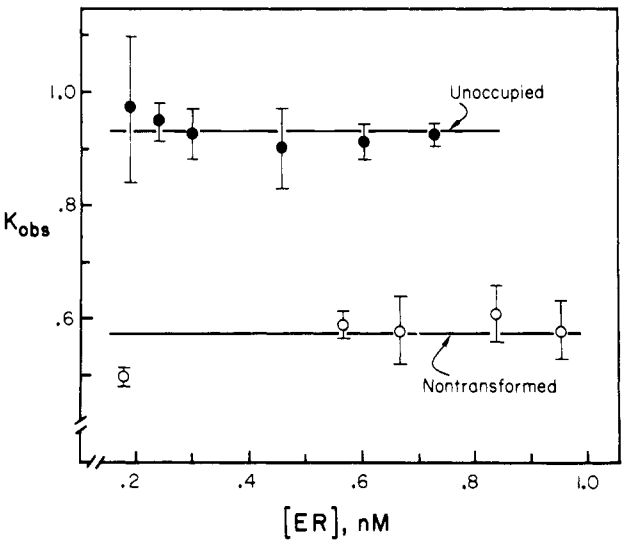

FIGURE 2: Partition coefficients of unoccupied (●) and nontransformed (○) ER as a function of receptor concentration in TED/0.1 M Li_2SO_4 systems. Tubes containing 5.4% (w/w) of both dextran (M_r 510000) and poly(ethylene glycol), 0.1 M Li_2SO_4, and varying amounts of rat uterine cytosol were partitioned at pH 8.2 (●) or 7.8 (○) and assayed as described under Materials and Methods. For both ER forms, the final ER concentration was manipulated by diluting concentrated cytosol with buffer such that all other cytosolic components were being equally altered. ER concentrations are expressed as the total dpm of ER recovered after phase partitioning per total phase H_2O. Lines represent the mean of all K_{obsd} values for each ER form.

The cross-point analysis of unoccupied and nontransformed ER is shown in Figure 3. ER was partitioned in 0.1 M NaCl, 0.3 M LiCl, and 0.1 M Li_2SO_4 over the pH range of 4.5–7.2. Lowering the partitioning pH below 8.0 reduces the salt-dependent differences in K_{obsd} for both ER forms (Table II) until the pH profiles in all three salts intersect at discrete points on both axes. The values at the points of intersection indicate a p*I* of ~5.5 for both forms of the protein. However, the K_0 of the unoccupied receptor is over twice that of the nontransformed ER. This difference in K_0 translates into a difference in standard free energies of transfer of ~400 cal/mol (eq 2). The pH dependence of both ER forms is well behaved in that the relative effect of salts on K_{obsd} below the p*I* is opposite of that seen above the p*I*. This is what one would expect if $\Delta\psi$ is unchanged but the sign of *Z* changes from negative to positive.

Effect of pH on ER Recovery. A plot of ER recovery vs. pH (Figure 4) supports the observation that the electrostatic properties of unoccupied and nontransformed ER are similar. Somewhat surprisingly, the plot is characteristic of a titration curve. Unoccupied and nontransformed ER recoveries are superimposable, with a 50% value at about pH 5.8. This loss of ER binding capacity below pH 6.5 is consistent with an

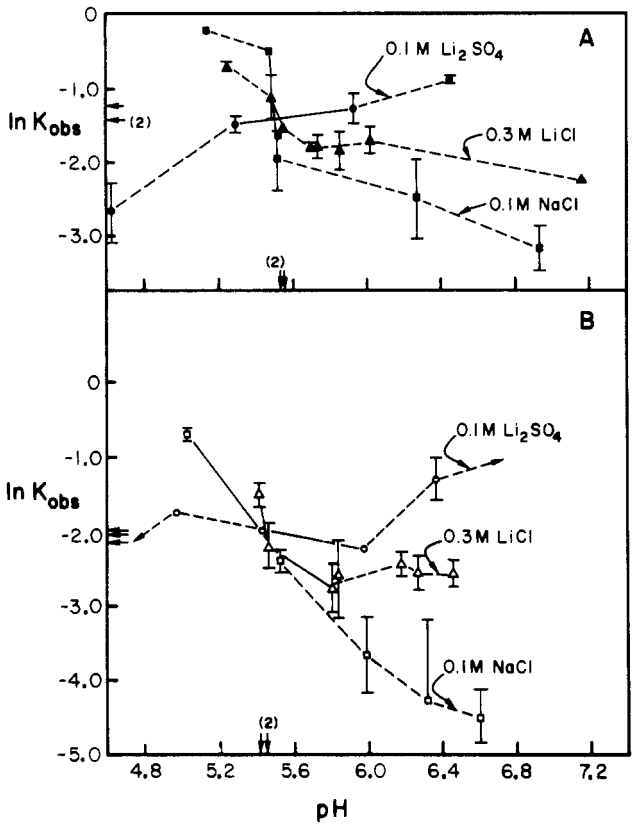

FIGURE 3: "Cross-point" analysis of unoccupied (A) and nontransformed (B) rat uterine estrogen receptor. Tubes containing either unoccupied (A) or nontransformed (B) ER, 5.4% of both dextran (M_r 510 000) and poly(ethylene glycol), and either 0.1 M NaCl (▲, △), 0.3 M LiCl (■, □), or 0.1 M Li_2SO_4 (●, ○) were partitioned at the indicated pH and otherwise treated as described under Materials and Methods. Manipulation and determination of partitioning pH are described under Materials and Methods. All systems were buffered with TED. Each point represents the mean ± the standard error of one K_{obsd} derived from duplicate partitionings (nk = 2). If no error bars are shown, the standard error is smaller than the data point. Arrows indicate the points of intersection of each salt pair on each axis. The K_0 of the unoccupied ER under these conditions is 0.25 ± 0.03, and that of the nontransformed ER is 0.12 ± 0.02. Both K_0 values represent the mean ± standard error of the three points of intersection on the K_{obsd} axis. The pI of the unoccupied ER is 5.55 ± 0.15, and that of the nontransformed ER is 5.45 ± 0.15. These values represent the mean of the three points of intersection on the pH axis ± the combined standard errors of the points of intersection and the pH determinations.

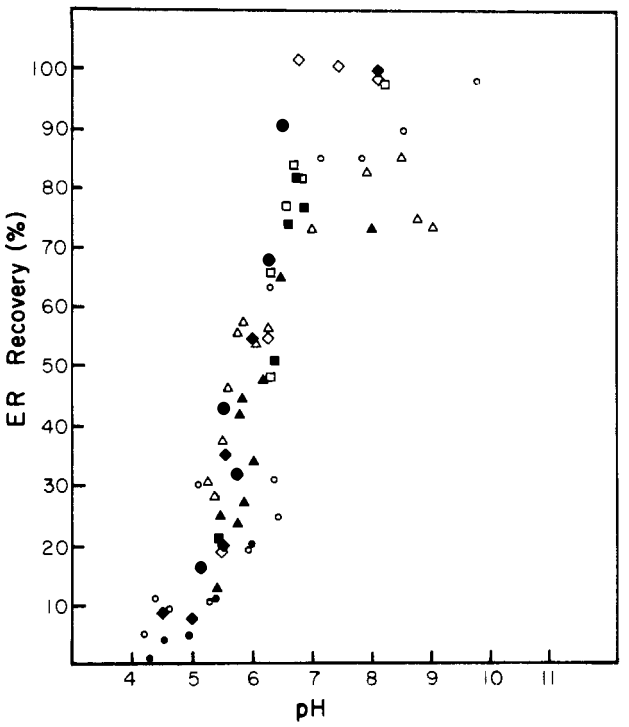

FIGURE 4: Recovery of unoccupied (closed symbols) and nontransformed (open symbols) estrogen receptor vs. partitioning pH. Points are derived from the same partitionings as shown in Figure 3 plus one experiment in 0.05 M K_2SO_4 and represent the amount of ER recovered at the corresponding partitioning pH. Symbols indicate partitionings that were performed in TED with either 0.1 M NaCl (▲, △), 0.3 M LiCl (■, □), 0.1 M Li_2SO_4 (●, ○), or 0.05 M K_2SO_4 (♦, ◇).

increased susceptibility to denaturation during mixing as the receptor nears its pI. A comparison of the recovery of ER in 0.1 M LiCl (Table III) and in 0.3 M LiCl indicates that elevated salt concentration reduces ER recovery by ~25% at pH 8.

Discussion

Our analysis of the pH and salt dependences of unoccupied and nontransformed ER partition coefficients has allowed us to determine the K_0 and pI of both of these receptor forms (Figure 3). The finding that both unoccupied and nontransformed ER have the same pI but a significantly different K_0 suggests that these ER forms differ in conformational but not electrostatic properties. Although the theory that determines the characteristic K_0 value of a protein is complex, a dominant factor is the net degree of hydrophobicity of the surface of the protein (Albertsson, 1971; Zaslavsky, 1982). At the pI, where the partition coefficients only represent receptor–solvent interactions in each phase, both unoccupied and nontransformed ER greatly prefer the lower dextran-rich phase. Considering that the lower phase is only slightly more hydrophilic than the upper phase, the very low K_0 values indicate that both ER forms are quite hydrophilic proteins. However, the fact that the unoccupied ER prefers the upper phase relative to the nontransformed ER indicates that the unoccupied ER possesses a hydrophobic domain that is less apparent on the nontransformed ER. On the basis of the findings that glucocorticoid binding to the glucocorticoid receptor is driven by hydrophobic interactions (Wolff et al., 1978; Eliard & Rousseau, 1984), it seems likely that this hydrophobic domain on the unoccupied ER may be the steroid binding site. Thus, our results indicate that estrogen binding to the unoccupied ER causes a considerable alteration in surface ER properties that precedes ER transformation and makes the ER less hydrophobic. Since this initial change in ER properties is similar in magnitude to that caused by heat transformation (as judged by the Δlog K_{obsd} value of the two transitions), it seems most likely that the different K_0 values of unoccupied and nontransformed ER are due to some type of conformational rearrangement. However, at this time, one cannot exclude the possibility that some or all of the difference in partition coefficients is due simply to estradiol binding (without any further change in ER properties). In this case, the portion of the steroid interacting with the solvent after binding must be considerably less hydrophobic than the unoccupied binding site, which we consider extremely unlikely. The functional relevance of this initial ligand-induced change in ER properties remains to be elucidated.

The effects of pH and salts on ER partition coefficients also serve as a test of whether the thermodynamic theory governing phase partitioning can be used to describe ER partitioning in crude cytosols. Taken together, the pH and salt dependences of unoccupied and nontransformed ER K_{obsd} values (Table II and Figure 3) indicate that ER partition coefficients can be

dramatically influenced by the combination of the net ER charge and a salt-induced interfacial potential across the phase boundary. The fact that in Figure 3 the unoccupied and nontransformed ER cross-points on both axes are essentially identical suggests that the conformation and p*I* of both ER forms are not affected by the different salts and by up to 0.3 M salt concentrations used in these studies, despite the fact that these same conditions have variable effects on receptor recovery. Ultimately, the cross-point analysis indicates that eq 4 can be used to interpret ER partitioning data obtained in pH and salt conditions which allow one to characterize both conformational and electrostatic surface properties of estrogen receptors in solution.

According to theory, the partition coefficient of any molecule should be independent of concentration unless concentration alters either the conformation or the net molecular charge of the molecule being partitioned. Thus, while the partitioning of many proteins in ATPP systems is concentration independent (Albertsson, 1971), ATPP has also been used to study the concentration-dependent dimer $\rightleftarrows$ tetramer transitions of hemoglobin (Middaugh & Lawson, 1980) and many other protein–protein associations (Backman, 1981; Chiang, 1981; Petersen, 1978), presumably because the charge and (or) K_0 of either a homogeneous or a heterogeneous aggregate differs from that of its monomeric components. Since the partition coefficient of the unoccupied ER cannot be lowered to that of the nontransformed ER by dilution to as low as 30 pM (essentially the detectable limit), we conclude that the difference in their partition coefficients is not due to differences in states of aggregation. From studies of binding cooperativity (Notides et al., 1981; Sakai & Gorski, 1984), it is known that the rat and calf uterine ER are normally monomeric below ~0.5 nM ER in dilute solution environments. However, it should be noted that many proteins can be forced to aggregate in the presence of dextran and PEG due to volume exclusion effects (Minton, 1983). Nonetheless, the stable K_{obsd} of the unoccupied ER at 30 pM indicates that unless the activity coefficient of the receptor is increased by 10–15-fold in the presence of the polymers (i.e., the ER *activities* are >0.5 nM), these receptor forms partition as monomers. This conclusion is also supported by the finding that the K_0 of both ER forms is independent of salt concentrations up to at least 0.3 M, a concentration which should at least partially disrupt aggregated ER. Andreasen (1982) has reached a similar conclusion regarding the monomeric state of the glucocorticoid receptor in ATPP systems with low receptor concentration. From a technical point of view, the concentration independence of ER partition coefficients allows the K_{obsd} to be easily reproduced, despite differences in receptor content of cytosol preparations and despite ER losses due to pH and ionic strength.

In conclusion, our initial applications of aqueous two-phase partitioning to the study of rat uterine estrogen receptors have yielded two important results. First, we have presented data that indicate that this technique can be used as a sensitive and reproducible in vitro probe of the properties and interactions of unoccupied steroid receptors. This in turn will allow one to investigate in vitro the molecular basis for the nuclear localization of native unoccupied steroid receptors (Welshons et al., 1984, 1985). Second, our finding that the conformational properties of the unoccupied ER monomer are significantly different than those of the nontransformed ER monomer indicates that steroid binding is a complex phenomenon, consisting of a temperature-independent alteration on the surface of the receptor that precedes the changes in ER properties associated with receptor transformation. Furthermore, this observation indicates that nontransformed estrogen receptors do not entirely reflect the native unoccupied ER state as has been frequently assumed throughout the literature.

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Purification and Characterization of Protein Synthesis Initiation Factor eIF-4E from the Yeast *Saccharomyces cerevisiae*†

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ABSTRACT: A 24000-dalton protein [yeast eukaryotic initiation factor 4E (eIF-4E)] was purified from yeast *Saccharomyces cerevisiae* postribosomal supernatant by m^7GDP–agarose affinity chromatography. The protein behaves very similarly to mammalian protein synthesis initiation factor eIF-4E with respect to (i) binding to and elution from m^7GDP–agarose columns and (ii) cross-linking to oxidized reovirus mRNA cap structures. Yeast eIF-4E is required for translation as shown by the strong and specific inhibition of cell-free translation in a yeast extract by a monoclonal antibody directed against yeast eIF-4E.

The binding of eukaryotic ribosomes to mRNA is a multistep process involving mRNA cap recognition, scanning of the 5′ nontranslated region, and AUG start codon selection [for reviews, see Safer & Anderson (1978), Revel & Groner (1978), Kramer & Hardesty (1980), Maitra et al. (1982), and Perez-Bercoff (1982)]. These steps are catalyzed by 6 or more eukaryotic initiation factors (eIF's)[1] composed of a total of about 15 polypeptides. One of the earliest reactions, the recognition of the mRNA cap structure by the ribosome, is mediated by the factors eIF-4A (Grifo et al., 1982; Edery et al., 1983), eIF-4E (also termed 24K-CBP or CBP-I; Sonenberg et al., 1978, 1979), eIF-4F (also termed CBP complex or CBP-II; Tahara et al., 1981; Edery et al., 1983; Grifo et al., 1983), and very likely eIF-4B (Grifo et al., 1982; Edery et al., 1983). It requires the hydrolysis of ATP (Kozak, 1980) and may involve melting of RNA secondary structure in the 5′ proximal region (Sonenberg et al., 1981, 1983). Cap recognition is a regulatable step as has been clearly demonstrated for poliovirus-infected HeLa cells, where the factor eIF-4F is inactivated early in infection leading to a shut-off of host mRNA translation [for a review and references, see the introduction to Sonenberg et al. (1983)].

At present, we know very little about the functions of individual initiation factors and their subunits in cap recognition and cap binding and how their activities are regulated in uninfected cells. To answer these questions, we have begun to study mRNA binding to ribosomes in the yeast *Saccharomyces cerevisiae* where biochemical approaches can be combined with powerful genetic approaches. Here, we report on the isolation from *S. cerevisiae* of one of the factors involved in mRNA cap recognition, the initiation factor eIF-4E.

Experimental Procedures

Yeast Strains. The strain *S. cerevisiae* VdH2 used for the purification of eIF-4E was purchased from Clipfel and Co., Rheinfelden, Switzerland. The strain GRF-18 (α, Leu 2–3, 2–112, His 3–11, 3–15) used for the preparation of cell-free translation extracts and total RNA was obtained from Dr. A. Hinnen, Ciba Geigy AG, Basel, Switzerland.

Purification of Yeast Initiation Factor eIF-4E. (*A*) *Preparation of Postribosomal Supernatant.* One kilogram of *S. cerevisiae* VdH2 was suspended in 2 L of 50 mM phosphate buffer, pH 7.0, 150 mM KCl, and 1 mM EDTA and kept at 20 °C overnight with aeration and stirring. $Mg(OAc)_2$ and PMSF were added to 5 and 1 mM, respectively, final concentrations, and the cell suspension was cooled to 0 °C in ice/water. The cells were broken with glass beads (0.4-mm diameter) in an ethanol/dry ice cooled Dyno-Mill at 3000 rpm at a pumping speed of 7 L/h. The homogenate was centrifuged in a Sorvall GS3 rotor for 10 min at 8500*g* at 2 °C. From this supernatant, mitochondria were pelleted by centrifugation in a CEPA centrifuge at 60000*g* at 2 °C with a flow rate of 30 mL/min. To the postmitochondrial supernatant was added KCl to a final concentration of 0.5 M, and ribosomes were pelleted by centrifugation in type 35 rotors at 105000*g* for 19 h at 2 °C. The postribosomal supernatant (ca. 2 L) was frozen in aliquots at −70 °C.

(*B*) *Affinity Chromatography on m^7GDP–Agarose.* All steps were performed at 0–4 °C essentially as described by Edery et al. (1983). Fifty milliliters of postribosomal supernatant (750 mg of protein) was diluted to 150 mL with buffer A (20 mM Hepes–KOH, pH 7.5, 0.2 mM EDTA, 0.5 mM PMSF, 7 mM β-mercaptoethanol, and 10% glycerol) and added to 1 mL of m^7GDP–agarose [m^7GDP was coupled to adipic acid dihydrazide–agarose (from P-L Biochemicals)] by

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[1] Abbreviations: eIF, eukaryotic initiation factor; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; kDa, kilodalton(s); Cl_3CCOOH, trichloroacetic acid; CBP, cap binding protein; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid.

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