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A MOLECULAR AND KINETIC ANALYSIS OF ESTROGEN RECEPTOR TRANSFORMATION*

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SUMMARY

Recent studies of the uterine estrogen receptor indicate it is a multi-subunit protein. Estradiol and temperature induce a conformational change in the 4 S estrogen-binding protein (EBP) or monomer (mol. wt. 7–8 × 10^4) as indicated by the high energy of activation (19–21 kcal mol^{-1}) accompanying the formation of the 4 S EBP dimer, 5 S EBP (mol. wt. 13–14 × 10^4). The formation of the 5 S EBP suggests that new intramolecular bonding forces have been established, presumably hydrophobic interactions, which were not available in the inactive 4 S EBP monomer. Kinetic analysis of the 4 S to 5 S EBP transformation indicates a second-order reaction, the dimerization of the 4 S EBP with a second (similar or dissimilar) subunit. The 5 S EBP produced by the *in vitro* warming of the cytosol-[^{3}H]-estradiol and the 5 S EBP extracted from isolated nuclei have similar molecular weights, sedimentation coefficients, molecular radii and rates of formation. These results suggest that an estradiol and temperature induced conformational change in the 4 S EBP leads to a macromolecular association process and receptor activation.

The uterine estrogen receptor is a soluble protein of the cytoplasmic (cytosol) fraction [1]. Following equilibration of the cytosol with [^{3}H]-estradiol, the receptor sediments during sucrose gradient centrifugation analysis in the presence of 0·4 M KCl as a 4 S estrogen-binding protein (EBP) [2]. In the presence of estradiol the estrogen receptor, by a temperature-dependent process, appears to translocate from the cytoplasm into the nucleus [3–5]. The nuclear form of the estrogen receptor following extraction with 0·4 M KCl and sucrose gradient analysis in 0·4 M KCl appears as a 5 S EBP [2, 4, 6]. Jensen and his associates have reported a cell-free "4 S to 5 S EBP transformation" of the receptor which produces a 5 S EBP similar to the isolated nuclear 5 S EBP [7, 8]. Unfortunately, considerable variation in the experimental conditions, sedimentation data, and its subsequent analysis among laboratories have left unresolved whether these variations are indicative of enzymatic changes [9], conformational changes [10], and specific or nonspecific associations of the EBPs [11–14], as well as their possible significance to estrogen action. This analysis of the 4 S to 5 S EBP transformation indicates a specific bimolecular association reaction which is invariably associated with receptor activation.

The molecular relationship between the 4 S EBP and the 5 S EBP

The transformation of the cytoplasmic 4 S EBP form of the estrogen receptor into a more rapidly sedimenting nuclear 5 S EBP form and the 5 S EBP produced by the *in vitro* incubation of cytosol-[^{3}H]-estradiol at an elevated temperature, as described by Jensen *et al.*[7, 8], may be the result of one of the following processes: (a) a conformational change of the 4 S EBP into a hydrodynamic form, which sediments more rapidly into the sucrose gradient; (b) a change in density (partial specific volume) of the 4 S EBP by the loss of a lipid, or the addition of carbohydrate and nucleotide moieties; or (c) an association of the 4 S EBP with another macromolecule resulting in an increased mass. Mechanism c is indicated by experimental data.

The molecular relationship between the 4 S EBP of the uterine cytosol and the 5 S EBP produced by the *in vitro* transformation procedure was assessed by comparisons of the molecular Stokes radii using gel chromatography and sedimentation coefficients using sucrose gradient centrifugation analysis in buffers containing 0·4 M KCl at pH 7·4. These molecular parameters and the Stokes–Einstein derivation of the Svedberg equation yield an estimation of the molecular weight and frictional ratio of the 4 S and 5 S EBPs [14, 15] (see Table 1).

The 4 S to 5 S EBP *in vitro* transformation results from an approximate doubling of the molecular weight, from 7–8 × 10^4 for the 4 S EBP to 13–14 × 10^4 for the 5 S EBP. The nuclear 5 S form of the estrogen receptor, extracted from uterine nuclei of immature rats administered [^{3}H]-estradiol, shows molecular parameters identical with the *in vitro* transformed EBP, *i.e.* a sedimentation coefficient of 5·5 S and a molecular Stokes radius of 59 Å. The densities of the 4 S and 5 S EBPs were not discernibly different, as measured by their relative rates of migration into very dense (38–50%) sucrose gradients for 20, 60, and 90 h. Preliminary studies have not revealed any distinguishing difference between the 4 S EBP and its putative complementary subunit necessary for 5 S

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Table 1. Molecular parameters of the estrogen receptor from gel chromatography and sucrose gradient analysis

Buffers	Sedimentation coefficient (S)	Molecular Stokes radius (Å)	Molecular weight	Frictional coefficient (f/f_0)
Untransformed receptor				
TEK	4·2 ± 0·04	44·0 ± 0·4	76,200 ± 4200	1·45
TEK-3 M urea	3·6 ± 0·04	53·8 ± 0·9	79,900 ± 3400	1·75
HEK (pH 6·8)	4·7 ± 0·04	43·2 ± 0·06	83,700 ± 3500	1·38
Transformed receptor				
TEK	5·5 ± 0·02	58·5 ± 0·5	132,700 ± 5100	1·60
TEK-3 M urea	4·6 ± 0·09	70·6 ± 1·0	133,900 ± 8200	1·93

The buffers were: TEK, 40 mM Tris, 2 mM EDTA, and 400 mM KCl at pH 7·4; HEK, 40 mM HEPES, 2 mM EDTA, and 400 mM KCl at pH 6·8. The method of Siegel and Monty[15] was used to estimate the molecular weight; the partial specific volume was assumed to be 0·725 $cm.^3$ per g for both the 4 S and 5 S EBP; data from Notides and Nielsen[14]. The values given are the mean ± standard error of the mean.

EBP formation [16]. Isolation and structural characterization may be necessary to determine whether the 5 S EBP consists of two similar or dissimilar subunits.

Aside from the 4 S to 5 S EBP transformation, which is due to an increase in mass by an association process, the 4 S EBP and the 5 S EBP show marked changes in their hydrodynamic properties, suggesting that they are capable of a high degree of conformational mobility or change without loss of [^{3}H]-estradiol-binding activity. The 4 S EBP and 5 S EBP in buffer containing 0·4 M KCl and 3 M urea at pH 7·4 show a decrease in their sedimentation coefficients and a reciprocating increase in their molecular Stokes radii; nevertheless, their molecular weights remain constant (Table 1). This conformational change is not seen with standard proteins under identical conditions. The relationship, if any, of the propensity of the estrogen receptor to change its conformation and its biological action remains to be resolved.

The variability of sedimentation coefficients of the estrogen receptor may be caused by one of several molecular interactions. (a) The 4 S to 5 S EBP transformation is a dimerization of the 4 S EBP with a second (similar or dissimilar) subunit of the receptor. Unlike the untransformed state (indicated by the 4 S EBP) the transformed 4 S EBP (*i.e.* the 5 S EBP) is not dissociated by high salt. (b) The untransformed receptor, the 4 S EBP, and the 5 S EBP will appear as an 8 S EBP using sucrose gradient analysis in the absence of KCl. Although this may suggest that the 4 S and 5 S EBPs are capable of forming weak associations with other cellular proteins at low ionic strength [11, 13], self-association of 4 S or 5 S EBP subunits should also be considered. (c) Changes in the molecular Stokes radius and sedimentation coefficient of the EBPs, *e.g.* caused by urea or pH, may reflect conformational changes. (d) Endogenous uterine proteases can produce several slowly sedimenting fragments of the estrogen receptor [9].

Kinetic analysis of the in vitro *4 S to 5 S EBP transformation*

A kinetic analysis of the 4 S to 5 S transformation shows it is a second-order reaction suggesting an estradiol- and temperature-activated dimerization. The uterine cytosol is preincubated with an excess of [^{3}H]-estradiol, thereby eliminating the unliganded 4 S EBP from kinetic consideration. The rate of 4 S to 5 S EBP transformation was plotted as the reciprocal of the 4 S EBP concentration vs the time of incubation at an elevated temperature. The transformation is essentially stopped by cooling to 0°C. The rate of 4 S to 5 S EBP transformation conforms to the integrated second-order rate equation

$$\frac{1}{4\,\mathrm{S\,EBP}} - \frac{1}{(4\,\mathrm{S\,EBP})_{t_0}} = k_a t$$

where the intercept $1/(4\,\mathrm{S\ EBP})_{t_0}$ is the initial concentration of the 4 S EBP; the slope k_a is the second-order rate constant in $M^{-1}\,min^{-1}$ [17]. The linear function indicates that the 4 S to 5 S EBP transformation is a second-order reaction of either a dimerization of the two 4 S EBPs or of a 4 S EBP with a second dissimilar subunit which must be present at a concentration equal to that of the 4 S EBP. The second-order rate constant at 28°C in the presence of 0·4 M KCl is $2 \times 10^7\,M^{-1}\,min^{-1}$ and is independent of the initial 4 S EBP concentration (Fig. 1a). These data plotted as the log 4 S EBP concentration vs time yield a non-linear function, verifying that it is not a first-order process.

A qualitative change in the bimolecular reaction of the 4 S to 5 S EBP transformation is observed in the absence of KCl or in the presence of 0·1 M KCl. An anomalous increase in the apparent second-order rate constant is observed with decreasing concentration of the initial 4 S EBP (Fig. 1b), indicating that an "inhibitor" is weakly associated with the 4 S EBP, or its complementary subunit, in low ionic strength buffers; this does not occur in the presence of 0·4 M KCl. Nevertheless, dilution of the cytosol with buffers of low ionic strength favors dissociation of the "inhibitor" and increases the fraction of the 4 S EBP available for the temperature-activated dimerization. The inability of ammonium sulfate precipitation and Sephadex G-25 filtration of the uterine

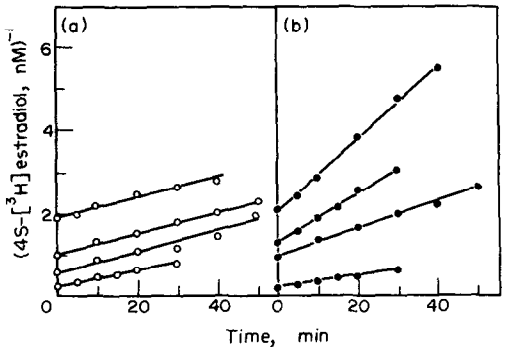

Fig. 1. The rate of the 4 S to 5 S EBP *in vitro* transformation plotted as the reciprocal of the 4 S EBP concentration. Uterine cytosol prepared in 40 mM Tris–1 mM dithiothreitol, pH 7·4, was equilibrated with 5 to 40 nM [^{3}H]-estradiol and 0·4 M KCl (A) or without KCl (B); then urea (1 M) was added and incubated at 28° for the times noted. The reaction was stopped by cooling to 0°; the excess [^{3}H]-estradiol was removed by adsorption to charcoal. The 4 S and 5 S EBPs were separated by sucrose gradient centrifugation analysis [14], and the concentrations of the 4 S to 5 S EBPs were determined with a Dupont 310 curve resolver.

cytosol to eliminate the anomalous second-order kinetics suggests it is not due to a low molecular weight substance. The "inhibitor" may reflect the tendency of the estrogen receptor to associate nonspecifically with other proteins [11], or the 4 S EBP may form weak associations with complementary subunits which must first dissociate from one another before reassociating in the proper conformation necessary for the formation of the 5 S EBP (Fig. 2).

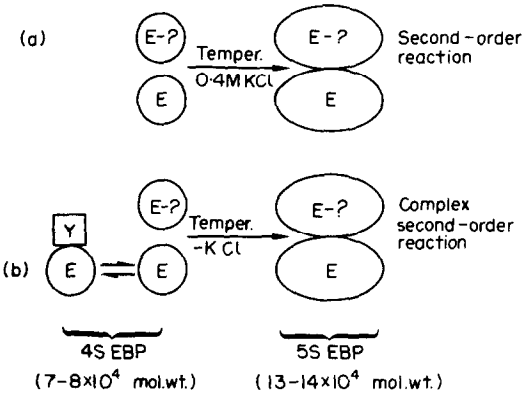

Fig. 2. The interactions of the estrogen-binding proteins during receptor transformation. In the presence of 0·4 M KCl the 4 S EBP after association with [^{3}H]-estradiol (E) dimerizes, due to a temperature-induced conformational change, with a second macromolecule (E-?) of identical or very similar molecular properties. The reaction is a simple second-order reaction (A). In buffers without or with 0·1 M KCl the second-order rate constant increases with dilution, indicating a predissociation of the 4 S EBP or its complementary subunit from a weak association with an "inhibitor" Y (B). The "inhibitor" Y is a macromolecule which may be the 4 S EBP, its complementary subunit, or some unrelated cellular protein. The weak associations of the 4 S EBP are not detected by sucrose gradient centrifugation analysis in 0·4 M KCl, so there appears a single population, the 4 S EBP, while the transformed 4 S EBP is not dissociated by 0·4 M KCl and appears as the 5 S EBP.

The rate of 4 S to 5 S EBP transformation is markedly temperature-dependent, showing a 200-fold increase from 0°C to 35°C. The Arrhenius energy of activation is 21·3 kcal mol^{-1} in buffer without, and 19·1 kcal mol^{-1} in buffer with, 0·4 M KCl. This unusually high energy of activation suggests that marked conformational changes are occurring during the temperature-activated receptor transformation. The similarity of the energy of activation for the 5 S EBP formation, in the presence of an excess of [^{3}H]-estradiol, and the absence or presence of 0·4 M KCl, suggests that the rate-limiting step of receptor transformation is not [^{3}H]-estradiol-binding or predissociation of the 4 S EBP from its weak association, as observed in low ionic buffers, but rather the formation of the 5 S EBP. The energy of activation observed in these *in vitro* receptor transformation studies is similar to the energy of activation (20·7 kcal mol^{-1}) associated with the formation of "specific estradiol-binding sites" in a uterine cell suspension study reported by Williams and Gorski[18]. The similarity of the energy of activation for this *in vitro* study to the intact uterine cell observation [18] supports the possibility that identical molecular processes are being measured by these two methods, although the data alone cannot be taken as proof of identity. The dissociation of the 5 S EBP to the 4 S EBP was effectively produced by 40 mM HEPES, 400 mM KCl, 3 M urea, pH 6·8, at 0°C. The dissociation is a first-order process with a half-time of 5–6 h.

In vivo *transformation of the estrogen receptor*

The rate of the 4 S to 5 S EBP *in vitro* transformation at 37°C at physiological ionic strength occurs very rapidly. The reaction is essentially completed (≥85% 5 S EBP) within 3–5 min in buffer containing 40 mM Tris, 150 mM KCl, 1 mM dithiothreitol, and 1 M urea at pH 7·4. Under *in vivo* conditions the rate of transformation appears more rapid than previously estimated. Recent experiments indicate that the concentration of the 4 S EBP recovered from the cytosol fraction after administration of [^{3}H]-estradiol to immature rats, and particularly following *in vitro* incubation of surviving uteri, has been overestimated due to an experimental artifact. Surviving uteri incubated for 15 to 60 min at 37°C with 5 nM [^{3}H]-estradiol were rinsed well in buffer and homogenized in buffer without or with 1 μM unlabeled estradiol. Sucrose gradient centrifugation analysis of the cytosol fraction in 0·4 M KCl showed that much less 4 S EBP [^{3}H]-estradiol was recovered when homogenized in the presence of unlabeled estradiol (Fig. 3a). This observation suggests that a large fraction of the 4 S EBP [^{3}H]-estradiol complex was formed with [^{3}H]-estradiol nonspecifically associated with the uterine surface or cell during homogenization at 0°C [19].

Nevertheless, when the corresponding nuclear fractions were washed with buffer and extracted with buffer containing 0·4 M KCl (without and with 1 μM

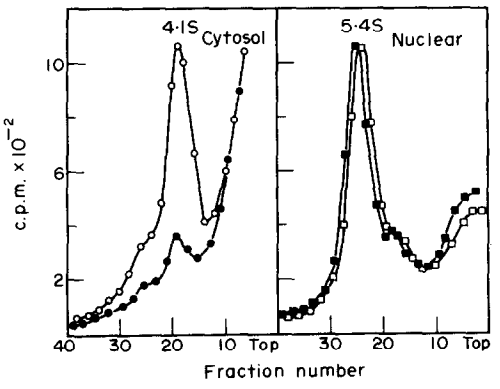

Fig. 3. Sucrose gradient centrifugation analysis of the uterine cytosol and nuclear fractions. Isolated uteri were transferred to an *in vitro* incubation medium containing 5 nM [^{3}H]-estradiol for 30 min at 37°. The uteri were rinsed in cold buffer, then homogenized in 40 mM Tris–2 mM EDTA, pH 7·4, buffer without or with unlabeled 1 μM estradiol. The nuclear pellet was washed twice with the dilute buffer, then extracted with the above buffers containing 0·4 M KCl. The samples prepared in buffer without unlabeled estradiol had 3·2 mg protein/ml (○) or 1·0 mg protein/ml (□); those prepared in buffer with 1 μM estradiol had 3·1 mg protein/ml (●) and 1·2 mg protein/ml (■).

estradiol), no difference was observed in the amount of [^{3}H]-estradiol associated with the nuclear 5 S EBPs. This suggests that the 5 S EBP [^{3}H]-estradiol was produced during the 37°C incubation of the uteri and not during the 0°C homogenization of the uteri (Fig. 3b). Concurrently, with the rapid formation of the 5 S EBP [^{3}H]-estradiol complex, the 5 S EBP associates with the nucleus, while *in vivo* the cytoplasm at any given time contains a minimal number of receptors associated with [^{3}H]-estradiol.

The amount of [^{3}H]-estradiol nonspecifically associated with the 4 S EBP during homogenization in buffers without unlabeled estradiol can also be detected in surviving uteri incubated in medium containing 0·1 nM [^{3}H]-estradiol or following the *in vivo* administration of 0·1 μg of [^{3}H]-estradiol. The preincubation of the 4 S EBP [^{3}H]-estradiol with 1 μM unlabeled estradiol at 0°C does not eliminate the appearance of the 4 S EBP [^{3}H]-estradiol complex during sucrose gradient centrifugation analysis, which confirms that the observed decrease in the radioactivity is not simply due to an exchange with unlabeled estradiol.

These uterine studies indicate that the [^{3}H]-estradiol forms a complex with the 4 S EBP that is rapidly transformed into the 5 S EBP, at a rate of transformation comparable to that observed *in vitro*. The 5 S EBP is found associated with the nucleus; the actual concentration of 4 S EBP [^{3}H]-estradiol in the cytoplasm is much less than previously estimated. Williams and Gorski, using a uterine cell suspension, have also observed the formation of nuclear-[^{3}H]-estradiol binding sites within a few minutes and at a more rapid rate than reported earlier [19, 20].

CONCLUSION

This analysis of the estrogen receptor indicates that an estradiol- and temperature-dependent change in the 4 S EBP (monomer) leads to a marked conformational change, as indicated by the high energy of activation and the formation of the non-salt-dissociable 4 S EBP dimer, the 5 S EBP. The formation of the 5 S EBP suggests that new intramolecular bonding forces have been established, presumably hydrophobic interactions, which were not available in the untransformed 4 S EBP monomer. The *in vitro* 5 S EBP produced by warming the cytosol-[^{3}H]-estradiol and the 5 S EBP extracted from isolated nuclei appear to have identical molecular weights, sedimentation rates, and gel filtration characteristics. They also appear to have in common a rapid rate of formation with similar energies of activation. These results suggest that this macromolecular association process, which correlates with receptor activation, is indicative of a change in the conformational relationship of the estrogen receptor's subunits.

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