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A MOLECULAR ANALYSIS OF THE HUMAN ESTROGEN RECEPTOR

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SUMMARY

The human myometrial estrogen receptor, isolated in buffer containing diisopropylfluorophosphate (DFP) to inhibit proteolytic activity, was partially purified by ammonium sulfate fractionation. Sucrose gradient analysis in dilute Tris buffer, pH 7.5, without KCl indicates that the cytoplasmic estrogen receptor sediments as an 8 S, 5 S and 4 S estrogen-binding protein (EBP). Isolation of the estrogen receptor without DFP yields predominantly a proteolytic fragment of the receptor which sediments as a 3 S EBP in gradients having low salt or high salt concentrations, and has a molecular weight of 30,000 to 40,000.

The nuclear form of the estrogen receptor has a sedimentation coefficient of 3.8 ± 0.04 S in sucrose gradients containing high salt concentrations, a molecular Stokes radius of 38.5 ± 0.9 Å, and a molecular weight of 60,000 to 70,000, values similar to the cytoplasmic form of the receptor. The cytoplasmic estrogen receptor shows a temperature-enhanced activation as indicated by its increased affinity for isolated uterine nuclei. In contrast to the activated estrogen receptor from the rat or calf uterus, the human uterus does not produce a 5 S EBP (a dimer of the 4 S EBP with another macromolecule) in high salt concentration at 0–4°C. In sucrose gradients containing 0.15 M KCl, centrifugation at 0, 6, 12, 20, and 25°C indicates that the human estrogen receptor undergoes a temperature dependent increases in its sedimentation coefficient from 4 S to 5 S and a transformation similar to that of the rat or calf estrogen receptor, but it is more readily dissociated by high salt or low temperature; the preferred form of the human estrogen receptor under more physiological conditions is the 5 S EBP dimer.

INTRODUCTION

Specific cytoplasmic estrogen receptors or estrogen-binding proteins (EBP) of high affinity and specificity present in the uterus of human [1–5] and other species [6, 7] have been described. The association of estradiol with the receptor results in its transformation or activation by a temperature dependent process [8, 9]. Consequently, the receptor translocates into the nucleus, where it binds to the chromatin and initiates RNA biosynthesis [10]. This general framework for the mechanism of action of the estrogens appears to be common to all steroid hormones [7]. Studies of the estrogen receptor from a number of species have shown, using sucrose gradient centrifugation analysis in buffer containing 0.4 M KCl, that concomitantly with the activation of the receptor, the cytoplasmic form of the estrogen receptor has a sedimentation coefficient of approx. 4 S while the transformed or nuclear form has a sedimentation coefficient of 5 S [11]. Nevertheless, a similar conversion in the sedimentation characteristics of other steroid hormone receptors following their activation has not been detected [7].

This communication describes the activation of the human myometrial estrogen receptor for which a 4 S to 5 S EBP transformation is not detected by the usual sucrose gradient methods. When sucrose gradient centrifugation conditions are modified to more physiological concentrations of salt, pH, and temperature, the human estrogen receptor sediments predominantly as a 5 S EBP.

EXPERIMENTAL

[2,4,6,7-^{3}H]-Estradiol (97 Ci/mmol) was obtained from New England Nuclear Corp., and diisopropyfluorophosphate (DFP) was purchased from Sigma Chemical Co.

Normal uterine samples were obtained from patients immediately after hysterectomy for pelvic relaxation and were considered post-menopausal. The myometrial tissue sample (2–10 g) was excised and immediately placed in liquid nitrogen or in Eagle's Basal Medium (Grand Island Biological Co.), then reduced to powder with a steel mortar and pestle cooled with liquid nitrogen. The *in vitro* incubation of tissue slices has been described earlier [5]. The tissue was homogenized with a Polytron PT-10 (Brinkmann Instruments). The buffers used were 40 mM Tris–1 mM dithiothreitol, pH 7.5 (TD) or TD plus KCl at the concentrations cited. The homogenate was centrifuged at 220,000 ***g*** for 1 h and provided a supernatant referred to as the "cytosol."

Partial purification of the estrogen receptor was obtained by slowly adding a saturated solution of ammonium sulfate, in TD buffer pH 7.5 at 0°, to the cytosol until 30% saturation with respect to ammonium sulfate was reached. The precipitates were

dissolved in buffer, centrifuged clear, and desalted by Sephadex G-25 filtration in the buffer cited. Sucrose gradient centrifugation analysis, the protein standards, and the fractionation procedures have been described earlier [12]. Experimental details are noted in the legends. For the *in vitro* incubation of the receptor with uterine nuclei, the uterine nuclear isolation procedure of Buller *et al.* was used [13].

RESULTS

Sedimentation characteristics of the cytoplasmic estrogen receptor

The estrogen receptor from the human myometrium partially purified by ammonium sulfate fractionation sediments into sucrose gradients containing TD buffer without KCl as a 7.9 ± 0.07 S, 4.8 ± 0.15 S and 3.6 ± 0.11 S estrogen-binding protein (EBP), referred to respectively as the 8 S, 5 S, and 4 S EBP (Fig. 1). This is in contrast to the estrogen receptor from immature rat or calf uteri, which sediments only as an 8 S EBP under the identical conditions. We have previously described the presence of a trypsin-like enzyme in the human uterus, active at 0°C and neutral pH [14]. The propensity of the human estrogen receptor to form the 8 S, 5 S, and 4 S EBP oligomers in a low ionic strength buffer is probably not caused by this uterine protease since we have taken precautions to inhibit its activity. The human uterine tissue samples were immediately placed in liquid nitrogen following hysterectomy. Then the tissue was powdered in the presence of liquid nitrogen and homogenized in buffer containing 5 mM diisopropylfluorosphosphate (DFP), an inhibitor of the uterine protease. The DFP inhibition was effective immediately at 0°C, while the weaker protease inhibitors, e.g., tosyl lysine chloromethylketone or phenylmethysulfonyl fluoride, provided inhibition only after 30 to 60 min at 25° to 30°C.

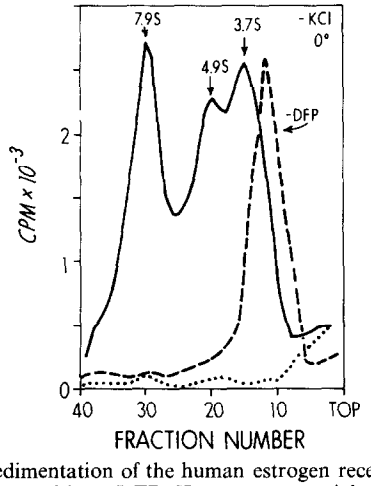

Fig. 1. Sedimentation of the human estrogen receptor isolated with or without DFP. Human myometrial tissue was homogenized in TD buffer with 5 mM DFP and the cytosol equilibrated with 5 nM [^{3}H]-estradiol (——) or 5 nM [^{3}H-estradiol plus 500 nM unlabeled estradiol (....). Cytosol prepared without DFP was equilibrated with 5 nM [^{3}H]-estradiol (----). The 30% ammonium sulfate precipitates of the cytosols were dissolved in TD buffer and centrifuged into 10–30% sucrose gradients in TD buffer for 18 h at 50,000 rev./min, 0°C.

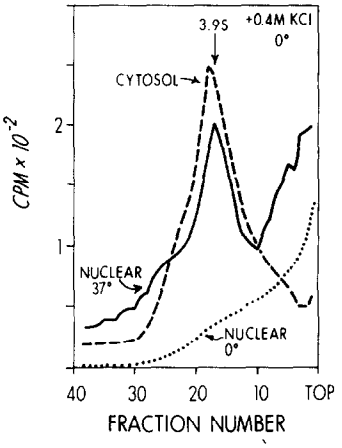

Fig. 2. Sedimentation of the human nuclear and cytoplasmic estrogen receptors. Myometrial tissue slices were incubated for 30 min at 37°C or 0° in Eagle's medium containing 1 nM [^{3}H]-estradiol and 1 mM DFP. The tissue was rinsed and homogenized in 40 mM Tris—2 mM EDTA, pH 7.5, containing 5 mM DFP and 10^{-6} M unlabeled estradiol. The nuclear pellet was washed twice in the above buffer, then extracted with 40 mM Tris—2 mM EDTA—0.5 M KCl, pH 8.5, for 1 h at 0°C. Sucrose gradient analysis was performed in 5–20% sucrose gradients with 40 mM Tris—2 mM EDTA—0.4 M KCl, pH 7.5; samples were centrifuged at 50,000 rev./min for 19 h at 0°C.

Centrifugation analysis of the cytosol estrogen receptor in salt-containing gradients, isolated in the presence of DFP and followed by ammonium sulfate purification, revealed a single peak with a sedimentation coefficient of 3.8 ± 0.04 S (Fig. 2). Sephadex G-200 gel chromatography indicated that in the presence of TD–0.4 M KCl buffer the estrogen receptor appeared as a single peak with a molecular Stokes radius of 38.5 ± 0.9 Å (data not illustrated). The molecular weight of the salt-dissociated 4 S EBP, calculated by the method of Siegel and Monty [15] was 60,000 to 70,000 daltons, slightly smaller than the salt-dissociated 4 S EBP of the rat uterus—70,000 to 80,000 daltons [12].

The isolation of the estrogen receptor from the human myometrium in buffers without DFP generally revealed a single [^{3}H]-estradiol-binding protein with a sedimentation coefficient of 3.1 ± 0.1 S (Fig. 1). The range of the sedimentation coefficients observed for this proteolytic fragment of the estrogen receptor was 3.7 S to 2.9 S, with a molecular Stokes radius of 26.7 ± 0.4 Å and a molecular weight of 35,000 to 40,000 [5]. This proteolytic fragment of the receptor had retained its estradiol-binding activity, although it was incapable of forming the 8 S oligomer protein.

Infrequently, myometrial tissue samples prepared in buffer without DFP simultaneously showed the 8 S

EBP and the 3 S proteolytic fragment. The presence of the 8 S EBP appears to correlate with tissue samples having lower trypsin-like activity, i.e., lower capacity to hydrolyze substrates of trypsin, such as benzoyl arginine p-nitroanilide [14]. The ammonium sulfate fractionation also served to separate the receptor from the protease, since the receptor is precipitated in the 0 to 30% fraction and the protease is in the 40 to 70% fraction [5], and thereby prevented formation of the 3 S EBP proteolytic fragment.

The 8 S, 5 S and 4 S EBP isolated in the 0 to 30% ammonium sulfate fraction have a high affinity with a low capacity for binding estradiol, indicating that this fraction contained the estrogen receptor. The cytosol from myometrial tissue that was homogenized in buffer containing DFP was equilibrated with 5 nM [^{3}H]-estradiol plus 500 nM unlabeled estradiol. The 30% ammonium sulfate fraction showed no estradiol-binding proteins, suggesting that the 30% ammonium sulfate fractions contained the estrogen receptor with little or no high capacity nonspecific estradiol-binding proteins (Fig. 1). The nonspecific estrogen-binding proteins, e.g., serum albumin, were found in the 50 and 70% ammonium sulfate fraction.

Thus, the human estrogen receptor shows a heterogeneous sedimenting pattern (i.e., 8, 5 and 4 S EBP) in KCl-free gradients which is not seen using uteri from other species. Although it cannot be entirely excluded that the sedimentation pattern is caused by a limited proteolysis of the receptor by proteolytic activity *in vivo* or during hysterectomy, these results were consistently observed in 10 different tissue samples prepared with buffer containing DFP and partially purified by ammonium sulfate fractionation.

Nuclear estrogen receptor and receptor transformation

The human nuclear estrogen receptor sediments as a 4 S receptor in sucrose gradients containing high salt, clearly slower than the 5 S from rat or calf nuclei but similar to the sedimentation of the cytoplasmic form of the receptor (Fig. 2). Myometrial tissue slices were incubated in Eagle's medium containing 1 nM [^{3}H]-estradiol and 1 mM DFP at 37°C for 30 or 60 min in order to translocate the estrogen into the nucleus while simultaneously inhibiting the uterine protease activity. Whether DFP is deleted from the incubation medium or present in the homogenization medium, the results are the same. Therefore the DFP is neither inhibiting the *in vitro* nuclear uptake of the estrogen receptor nor the 5 S EBP formation. Estrogen receptor uptake by the nucleus is temperature dependent. Myometrial tissue slices incubated at 0–4°C contained the cytoplasmic 4 S EBP, but not the nuclear 4 S EBP (Fig. 2). Gel chromatography in buffers containing high salt concentrations indicated that the estrogen receptor from the nuclear fraction of myometrial tissue incubated with estradiol at 37°C had a molecular Stokes radius of 38 Å, which is identical to the Stokes radius of the cytoplasmic 4 S EBP produced by high salt concentrations. Thus,

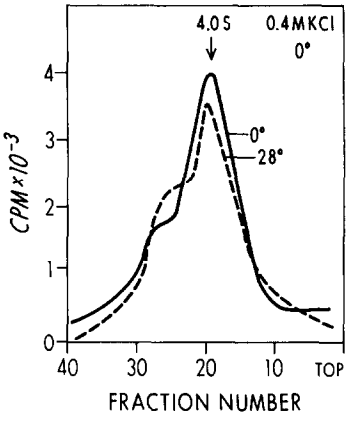

Fig. 3. *In vitro* incubation of the estrogen receptor at 0° and 28°C. Myometrial tissue was homogenized in TD buffer containing 5 mM DFP, then the cytosol was equilibrated with 10 nM [^{3}H-estradiol. The 30% ammonium sulfate precipitate of the cytosol was dissolved in TD—0.1 M KCl buffer. An aliquot was incubated at 0°C (——) or 28°C (----) for 30 min and then layered on a 5–20% surcrose gradient prepared with TD—0.4 M KCl buffer. The samples were centrifuged at 50,000 rev./min for 18 h, 0°C.

the 4 S EBP isolated from the nuclear fraction is not the 3–4 S proteolytic fragment of the receptor, which has a molecular radius of 28 Å.

The human cytoplasmic 4 S EBP does not show an *in vitro* transformation to the 5 S EBP. The myometrial cytosol, or the 30% ammonium sulfate fraction of the cytosol in TD – 0.1 M KCl buffer with 5 mM DFP, was made 1 M with respect to urea and incubated at 28°C for 30 min. These conditions produce a maximal amount of the 5 S EBP using the rat or calf estrogen receptor; nevertheless, an insignificant amount of 5 S EBP was observed following sucrose gradient analysis in the presence of 0.4 M KCl at 0°C (Fig. 3). The *in vitro* incubation of estrogen receptor at 28°C for 60 min or 35°C for 30 min, with

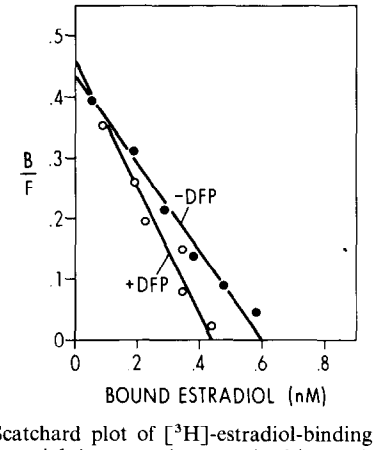

Fig. 4. Scatchard plot of [^{3}H]-estradiol-binding by cytosol. Myometrial tissue was homogenized in TD buffer with (○) 5 mM DFP (the cytosol contained 9.4 mg protein per ml) or without DFP (●) (the cytosol contained 9.0 mg protein per ml). Charcoal-Dextran was used to adsorb the free [^{3}H]-estradiol following equilibration.

or without the 1 M urea or 0.1 M KCl, did not significantly increase the quantity of the 5 S EBP observed.

Although the customary sucrose gradient condition did not detect a temperature-generated 4 S to 5 S transformation of the human estrogen receptor using tissue slices or the cytosol, the receptor was still capable of a temperature-enhanced activation as measured by an *in vitro* nuclear binding assay. Portions of a single myometrial tissue sample were homogenized in TD buffer with or without DFP. The [^{3}H]-estradiol-binding and nuclear-binding activities were measured. The [^{3}H]-estradiol-binding affinity and capacity of the cytosols, prepared with or without DFP, were essentially identical. The cytosols were equilibrated with 0.2 to 10 nM [^{3}H]-estradiol for 3 h at 0°C. The nonspecifically bound estradiol was determined with parallel incubations containing the addition of a 100-fold excess of diethylstilbestrol to the cytosol. The nonspecific binding was 10–15% of the total [^{3}H]-estradiol bound. The Scatchard analysis indicated that the cytosol prepared with DFP had a dissociation constant of 1 nM and a binding capacity of 0.45 nM for [^{3}H]-estradiol, while the cytosol prepared without DFP had a dissociation constant of 1.3 nM and a binding capacity of 0.6 nM (Fig. 4). Aliquots of the same myometrial cytosol samples (with or without DFP) that had been incubated with 5 nM [^{3}H]-estradiol (Fig. 4) were incubated for an additional 30 min at 0° and 28°C. The aliquots were then incubated with myometrial nuclei for 1 h at 0°C.

A greater quantity of the estrogen receptor, prepared in buffer containing DFP, bound to isolated uterine nuclei when preincubated at 28°C than at 0°C. The receptor prepared in the absence of DFP did not show any nuclear-binding activity, whether preincubated at 0° or 28°C (Fig. 5). The nuclear-binding activity of the estrogen receptor (with and without DFP) is identical whether using human or calf uterine nuclei.

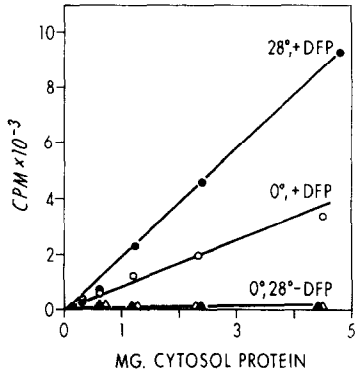

Fig. 5. Estrogen receptor binding to uterine nuclei. Samples of uterine cytosol prepared in TD buffer with and without 5 mM DFP were incubated with 5 nM [^{3}H]-estradiol, plus or minus 500 nM diethylstilbestrol. The cytosol samples were incubated at 0° or 28° for 30 min. Aliquots were incubated with myometrial nuclei for 1 h at 0°. The nuclei were washed with buffer and the radioactivity was measured. The data are the mean of duplicate determinations minus the non-specific binding.

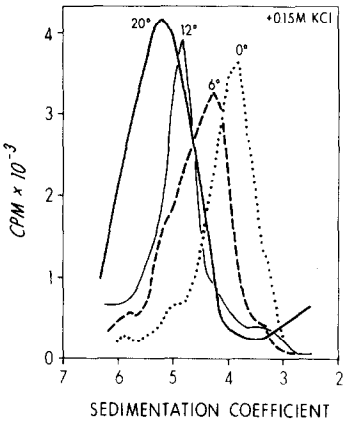

Fig. 6. Increases in the sedimentation rate of the estrogen receptor with increasing temperature. Myometrial tissue was homogenized in TD—150 mM KCl buffer containing 5 mM DFP. The cytosols were equilibrated with 5 nM [^{3}H]-estradiol, then made 30% saturated with respect to ammonium sulfate. The precipitate was dissolved in TD—0.15 M KCl buffer. The samples were centrifuged into 5–20% sucrose gradients in TD—0.15 M KCl buffer at the temperature cited.

Thus, studies using the human uterus require an effective protease inhibitor such as DFP to maintain the integrity of the receptor. In addition, sucrose gradient analysis under standard high salt conditions does not show a 4 S to 5 S EBP conversion associated with receptor activation.

Effect of temperature on the sedimentation coefficient of the estrogen receptor

The human estrogen receptor undergoes a monomer-dimer interconversion in which the 5 S EBP is the preferred conformation under more physiological conditions of salt and temperature during sucrose gradient analysis. Human uterine cytosol that had been prepared in buffers containing DFP and partially purified by ammonium sulfate fractionation, showed a 4 S [^{3}H]-estradiol-binding protein in gradients containing TD – 0.15 M KCl buffer at 0°C. As the temperature at which the centrifugation was carried out increased, the sedimentation coefficient of the [^{3}H]estradiol-binding protein increased while still appearing as a single peak (Fig. 6). The gradual shift in the sedimentation coefficient continued until at 20°C the [^{3}H]-estradiol-binding protein had a sedimentation coefficient of 5 S. Sucrose gradient centrifugation analysis at elevated temperatures in 0.4 M KCl demonstrated that a high salt concentration favors dissociation of the 5 S EBP dimer as does low temperature (0–4°C). Centrifugation analysis of the receptor that had been isolated in buffers without DFP produced the 3 S EBP which cannot undergo temperature-promoted dimerization in buffered gradients containing 0.15 M KCl (Table 1).

Table 1. Temperature dependence of the sedimentation rate of the human estrogen receptor. The cytosol samples were prepared as described in Fig. 6. The buffer was 40 mM Tris—1 mM dithiothreitol and the KCl concentration as cited; the pH was 7.5 at the temperature of centrifugation. In all tubes the [^{3}H]-estradiol-binding peak migrated to about the middle of the gradient; the reduced time of centrifugation reflects the increase in the transport process with temperature. All gradients contained *E. coli* alkaline phosphatase and ovalbumin as sedimentation markers

Temperature	Time of centrifugation	Sedimentation coefficient		
		Buffer + 0.15 M KCl		Buffer + 0.4 M KCl
		+ DFP	− DFP	+ DFP
°C	h	S		S
0	19	3.9 ± 0.04 (10)*	3.1 ± 0.1 (15)	3.8 ± 0.04 (9)
0	16	4.3 ± 0.06 (4)	—	—
12	12	4.8 ± 0.07 (14)	3.3 (3)	4.4 ± 0.04 (7)
20	10	5.0 ± 0.08 (12)	—	—
25	8	4.9 (2)	—	—

* Mean ± S.E.M. (number of determinations).

DISCUSSION

The characterization of the molecular properties of the human estrogen receptor is dependent upon inhibition of a neutral protease activity with diisopropylfluorophosphate (DFP). Without a potent protease inhibitor the resulting 3–4 S EBP proteolytic fragment of the receptor can erroneously be assumed to be the 4 S EBP salt-dissociated monomer of the receptor or the nuclear form of the receptor [1, 4]. Other investigators have noted that phenylmethylsulfonyl fluoride is ineffective in inhibiting the human uterine protease at 0°C [3]. Thus, the presence of an active protease in human tissue produces an added complexity which must be controlled to obtain meaningful data about the human uterine estrogen receptor.

The present experiments indicate that the molecular properties of the estrogen receptor from the human differ qualitatively from those of other species. The activated or nuclear form of the human estrogen receptor sediments into sucrose gradients containing 0.4 M KCl at 0–4°C as a 4 S EBP, identical with the cytoplasmic 4 S EBP; this is in contrast to other species, in which the activated estrogen receptor sediments as a 5 S EBP. The human estrogen receptor shares a property common to other steroid hormone receptors: whether extracted from the cytoplasm or from the nucleus, it sediments as 4 S steroid-binding protein in high salt-containing gradients [7].

Our previous studies have demonstrated that the 4 S EBP from the rat uterus has a molecular weight of 70,000–80,000 and by an estradiol and temperature-enhanced dimerization reaction associates with another macromolecule to form the 5 S EBP, which has a molecular weight of 130,000–140,000. The formation of the 5 S EBP is highly temperature dependent; the energy of activation is 20 kcal mol^{-1}, indicating that marked conformational changes of the receptor must have taken place during the activation process [12, 16]. The conversion of the rat uterine 4 S EBP to the 5 S EBP dimer appears to be a dimerization reaction with a very large equilibrium constant favoring the dimer. Once formed, the 5 S EBP dimer is not readily dissociated under the specific conditions of the analyses, i.e., during sucrose gradient centrifugation analysis in sucrose, 0.4 M KCl, pH 7.5 at 0° for 16–20 h. The structure and function of the human estrogen receptor is also strongly temperature-dependent, but the estrogen receptor from the human uterus appears to have a lower equilibrium constant for the dimerization reaction than does the estrogen receptor from the calf or rat uterus under identical analytic conditions: high salt concentrations in sucrose for long time periods at 0°C. Thus, under more physiological conditions, e.g., temperature, salt concentration, and pH, the human estrogen receptor exists as a 5 S EBP dimer and not as the dissociated 4 S EBP monomer.

Our analysis indicates that either lowered temperatures or buffers of higher salt concentrations for 18–20 h lead to dissociation of the 5 S EBP. The appearance of a single distinct peak at each temperature during centrifugation indicates that a rapid 4 S $\rightleftharpoons$ 5 S association–dissociation equilibrium is present (i.e., rapid relative to the time of centrifugation). Therefore, the observed sedimentation coefficient will be a value lying between the sedimentation coefficients of the monomer and the dimer, and will be dependent upon the concentrations of the reactants, temperature, pH, ionic strength, and the values of the equilibrium constants. The gradual increase in the sedimentation coefficient reported here is dependent upon temperature of centrifugation and not upon receptor concentration, since all sample concentrations were within a 2- to 3-fold range.

The molecular behavior and characteristics of the estrogen hormone receptor appear to be similar to a number of enzymes whose activity is modulated by a ligand-mediated oligomerization process. This type of enzyme regulatory mechanism does not appear to be common to any particular group of enzymes or

metabolic pathways [17–20] and is distinct from the subunit-subunit interactions of allosteric regulatory enzymes [21].

Thus, enzymes whose activity is regulated by ligand-mediated and temperature-dependent association-dissociation reactions may serve as useful models for understanding the structural properties in relation to the activities of the steroid hormone receptors. In addition, the molecular properties of the human estrogen receptor in comparison with the rat or calf uterine estrogen receptor provide an experimental model for investigating the biological significance of the interconversion between the 4 S and 5 S estrogen-binding proteins.

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DISCUSSION

Clark. The proteolytic break-down product that is found in the experiment without DFP will still bind estrogen but yet does not undergo transformation. Is that a permanent condition? Have you permanently incapacitated that unit from undergoing transformation?

Notides. If we incubate the human estrogen receptor, isolated without DFP, with nuclei at 0° for 1 h we observe essentially no nuclear binding. We have not incubated for longer time periods.

Clark. I was thinking of a mixing experiment to see whether you can convert it back to a sticky form i.e. a transformed condition again.

Notides. How would you suggest doing that?

Clark. Well, I'm not really sure. I can think of some crude ways of doing it. I'm wondering if there is something in the crude cytosol preparation which would convert it back to a form that would bind to the nucleus.

Notides. We saw no evidence that we could increase the sedimentation coefficient or molecular radius of the human estrogen receptor, isolated in the absence of DFP, by mixing experiments.

Clark. It would be very interesting to know if that is a permanent change.

Notides. I think so. The molecular weight of the receptor is clearly decreased, and we have shown that the human uterus contains a protease which we have characterized (*Endocrinology*, **93** (1973) 210).

Clark. Would you care to discuss your work in relationship to the work of B. O'Malley and B. Shrcader?

Notides. Our previous work described the molecular properties of the estrogen receptor and demonstrated that the active form of the rat uterine estrogen receptor is a 5*S* dimer. This human estrogen receptor data suggests a molecular basis why some receptors, such as the progesterone receptor, during the usual analytical conditions (e.g. sucrose gradient, 0.4M KCl at 0°), do not show a difference in the sedimentation behavior between the cytoplasmic and nuclear forms. The active conformation, the 5S dimer of most steroid hormone receptors may be maintained predominately by hydrophobic interactions between the monomers; which is a temperature-dependent bonding force. In retrospect, it appears that during activation the estrogen receptor from the rat uterus must establish hydrophobic and other bonding forces between the monomers which are not disturbed during sucrose gradient analysis.