Carter, P. (1990) in *Mutagenesis: A Practical Approach*, Chapter 1, pp 1-25, IRL Press, Oxford, U.K.
Carter, P., & Wells, J. A. (1987) *Science (Washington, D.C.) 237*, 394-399.
Carter, P., & Wells, J. A. (1988) *Nature (London) 332*, 564-568.
Carter, P., & Wells, J. A. (1990) *Proteins: Struct., Funct., Genet. 7*, 335-342.
Carter, P., Winter, G., Wilkinson, A. J., & Fersht, A. R. (1984) *Cell 38*, 835-840.
Carter, P., Nilsson, B., Burnier, J. P., Burdick, D., & Wells, J. A. (1989) *Proteins: Struct., Funct., Genet. 6*, 240-248.
Fastrez, J., & Fersht, A. R. (1973) *Biochemistry 12*, 2025-2034.
Fersht, A. R. (1985) *Enzyme Structure and Mechanism*, 2nd ed., Chapter 7, Freeman, New York.
Gutfreund, H., & Sturtevant, J. M. (1956) *Biochem. J. 63*, 656.
Matsubara, H., Kaspar, C. B., Brown, D. M., & Smith, E. L. (1965) *J. Biol. Chem. 240*, 1125-1130.
McPhalen, C. A., & James, M. N. G. (1988) *Biochemistry 27*, 6582-6598.
Miller, J. H. (1972) in *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Mizuno, K., Nakamura, T., Ohshima, T., Tanaka, S., & Matsuo, H. (1988) *Biochem. Biophys. Res. Commun. 156*, 246-254.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157-162.
Takagi, H., Moringa, Y., Ikemura, H., & Inouye, M. (1988) *J. Biol. Chem. 263*, 19592-19596.
Wells, J. A. (1990) *Biochemistry 29*, 8509-8517.
Wells, J. A., & Powers, D. B. (1986) *J. Biol. Chem. 261*, 6564-6570.
Wells, J. A., Ferrari, E., Henner, D. J., Estell, D. A., & Chen, E. Y. (1983) *Nucleic Acids Res. 11*, 7911-7925.
Wells, J. A., Cunningham, B. C., Graycar, T. P., & Estell, D. A. (1986) *Philos. Trans. R. Soc. Lond., A 317*, 415-423.
Wells, J. A., Cunningham, B. C., Graycar, T. P., & Estell, D. A. (1987a) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5167-5171.
Wells, J. A., Cunningham, B. C., Graycar, T. P., Estell, D. A., & Carter, P. (1987b) *Cold Spring Harbor Symp. Quant. Biol. 52*, 647-652.
Wilkinson, A. J., Fersht, A. R., Blow, D. M., & Winter, G. (1983) *Biochemistry 22*, 3581-3586.
Yang, M. Y., Ferrari, E., & Henner, D. J. (1984) *J. Bacteriol. 160*, 15-21.

Differential DNA Binding by Calf Uterine Estrogen and Progesterone Receptors Results from Differences in Oligomeric States†

D. F. Skafar*
Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan 48201
Received December 10, 1990; Revised Manuscript Received March 5, 1991

ABSTRACT: The studies presented here provided evidence that the calf uterine estrogen and progesterone receptors exhibit different DNA-binding properties in vitro as a result of having different dimerization constants. The affinity of the estrogen and progesterone receptors for DNA was measured by using isocratic elution from DNA-Sepharose. The hormone-free estrogen receptor had a 10-fold higher affinity for DNA than did the hormone-free progesterone receptor when measured at receptor concentrations of 6-12 nM and 180 mM KCl. No effect on DNA binding by binding progesterone to its receptor was detected. This contrasts with the increased affinity for DNA and increased number of ions released upon DNA binding exhibited by the hormone-bound estrogen receptor. Between 2 and 3 ions were released when the progesterone receptor and the diluted estrogen receptor bound DNA. These observations suggested the progesterone receptor was in the monomeric state, whereas the estrogen receptor was in the dimeric state at receptor concentrations of 6-12 nM. When the dimerization constant of the progesterone receptor was measured, the value of ≈7 nM obtained was 20-fold higher than the value of 0.3 nM reported for the estrogen receptor. This makes it likely the two receptors exist in different forms at the same concentration in vitro. It is also suggested the predominant form of the estrogen and progesterone receptors in vivo could differ.

The calf uterine estrogen and progesterone receptors are members of the steroid hormone receptor superfamily of gene-regulatory proteins [for review see Evans (1988)]. The transcription-regulation activity of these proteins in vivo is controlled by steroid binding. The estrogen receptor has the characteristic properties of an allosteric protein: it is a homodimer (Notides et al., 1985); it binds estradiol with a positive cooperative mechanism and with a maximum Hill coefficient of 1.6 (Notides et al., 1981); and hormone binding induces a conformation change in the receptor leading to an increased affinity for DNA (Skafar & Notides, 1985). The calf progesterone receptor, although less well studied, sediments on sucrose gradients as a 4S, monomeric species and a 6S, possibly dimeric species (Theofan & Notides, 1984). It also binds

† This research was supported by NSF Grant DCB 8716044 and Wayne State University.
* Address correspondence to this author at the Department of Physiology, Wayne State University School of Medicine, 540 E. Canfield, Detroit, MI 48201.

0006-2960/91/0430-6148$02.50/0

steroid with a positive cooperative mechanism: its maximum Hill coefficient, however, is 1.2 (Theofan & Notides, 1984).

The similar steroid-binding properties of the two receptors are a direct reflection of their homologous structures. Each has a carboxy-terminal hormone-binding domain, a central DNA-binding domain containing two "zinc finger" motifs, a hinge region, and an immunogenic amino-terminal domain (Evans, 1988). The homologous DNA-binding domains of the human estrogen and progesterone receptors exhibit 56% identity (Misrahi et al., 1987). Each receptor also binds in the dimeric form to its specific hormone response element (Kumar & Chambon, 1988; Tsai et al., 1988). The expected similarity of the DNA-binding properties of the receptors is contradicted, however, by the observation that the progesterone receptor is eluted from a pseudoaffinity ligand for the DNA-binding site of the receptors (Orange–Sepharose) at a lower salt concentration than the estrogen receptor (0.15 M KCl vs 0.4 M KCl) (Bond & Notides, 1987).

The apparent discrepancy between the homologous DNA-binding domains of the receptors with the observed difference in DNA-binding properties in vitro was investigated by isocratic elution of each receptor from DNA–Sepharose (deHaseth et al., 1977; Skafar & Notides, 1985). Earlier studies using this approach have shown that hormone binding induces a conformational change in the estrogen receptor which leads to an increased affinity for DNA (Skafar & Notides, 1985). In this study, the affinity of each receptor for DNA, the number of ions released upon binding each receptor to DNA, and the effect of steroid binding on these properties were determined. Unexpectedly, the results indicated the receptors were in different oligomeric states, and the dimerization constant of the progesterone receptor was then measured. The results presented here support the hypothesis that a difference in the dimerization constants of the receptors leads to the observed differences in their DNA-binding properties. The results also indicate, as described in the supplementary material, that the DNA concentration can shift the monomer–dimer equilibrium of a DNA-binding protein toward either the dimeric or the monomeric form.

Experimental Procedures

Materials

[1,2-^{3}H]Progesterone (40–60 Ci/mmol) and [6,7-^{3}H]estradiol (40–60 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Calf thymus DNA, highly polymerized, was obtained from Sigma Chemical Co. (St. Louis, MO) and was ethanol precipitated before use. Ammonium sulfate was ultrapure grade from ICN Biomedicals, Inc. (Cleveland, OH). Unlabeled steroids were obtained from Steraloids (Wilton, NH) or Sigma Chemical Co. (St. Louis, MO). Cyanogen bromide activated Sepharose was from Sigma Chemical Co. (St. Louis, MO). Charcoal (Norit-A) was obtained from fisher (Livonia, MI). All other chemicals were reagent grade.

Methods

Preparation of Estrogen and Progesterone Receptors. The partially purified estrogen receptor was obtained as described by Weichman and Notides (1977). Calf uteri obtained from a local slaughterhouse were trimmed, frozen, and stored at −70 °C. The frozen uteri were pulverized in a stainless steel mortar and pestle. The powder was homogenized in ice-cold 40 mM Tris, 1 mM dithiothreitol, and 0.1 mM EDTA, pH 7.4, containing 0.2 mM phenylmethanesulfonyl fluoride (TDE buffer). The homogenate was centrifuged at 27000*g* for 10 min. The supernatant was removed and centrifuged at 220000*g* for 45 min to obtain cytosol. Solid ammonium sulfate was added to 30% saturation. The precipitate was collected by centrifugation and stored at −70 °C. The partially purified progesterone receptor was prepared the same way, with the exceptions that cytosol was passed through a phosphocellulose column ($^1/_5$ the volume of cytosol) at maximal speed and the receptor was then precipitated from the breakthrough fraction by the addition of solid ammonium sulfate to 35% saturation.

Measurement of Affinity for Calf Thymus DNA. The affinity of each receptor for DNA was measured by using isocratic elution from DNA–Sepharose (DeHaseth et al., 1977; Skafar & Notides, 1985). Ammonium sulfate precipitates were redissolved in TDE buffer containing 50 mM KCl and desalted by gel filtration chromatography. The receptor was incubated on ice in the presence or absence of 20 nM [^{3}H]-estradiol or 40 nM [^{3}H]progesterone. The salt concentration was adjusted to the indicated value prior to loading onto a 1-mL (estrogen receptor) or 2-mL (progesterone receptor) DNA–Sepharose column. After collection of 15 fractions (2 mL each) in TDE buffer containing the indicated KCl concentration, TDE buffer containing 1 M KCl was used to elute the receptor from the column. All buffers contained 0.2 mg/mL ovalbumin. One-milliliter aliquots of each fraction were taken. For those experiments in which the receptor was not preincubated with steroid, 5 nM [^{3}H]estradiol or 10 nM [^{3}H]progesterone was added to each aliquot. The amount of receptor in each aliquot was then determined by measuring protein-bound [^{3}H]steroid according to the following modifications of the hydroxylapatite assay used in Skafar and Notides (1985). For the estrogen receptor, aliquots were incubated for 1 h at 25 °C with 100 μL of a 10% hydroxylapatite slurry (w/v, in TD buffer). For the progesterone receptor, the aliquots were incubated for 2 h at 0 °C; 100 μL of the 10% hydroxylapatite slurry was added during the second hour. Pellets were washed three times with 3 mL of TD buffer containing 50 mM KCl and transferred to scintillation vials by rinsing twice in 2 mL of Scintiverse E (Fisher).

Calculation of Binding Constants. The binding constant between the receptor and DNA was calculated as described by DeHaseth et al. (1977). The percentage of receptor bound to the column was graphed semilogarithmically as a function of the fraction number. The slope of such a graph yields a proportionality constant (k) that is related to the binding constant according to the equation $K_a = V/(kD)$, where V is the volume of each fraction and D is the number of binding sites for the receptor on the DNA. The number of binding sites was determined by measuring the phosphate content of the DNA–Sepharose as described below. The amount of phosphate was assumed to equal the amount of nucleotides. Each nucleotide was assumed to begin a new binding site, in accordance with the model of McGhee and von Hippel (1974) for the interaction of proteins and nonspecific DNA. Since total DNA was used, specific binding sites are not detectable because of the large excess of nonspecific DNA (Yamamoto & Alberts, 1974). There are 30 mg of dried DNA–Sepharose/1 mL of column. The binding constants reported here are lower than reported elsewhere because they are expressed in terms of moles of nucleotide, instead of moles of 15 bp binding sites as used in the earlier work (Skafar & Notides, 1985).

Equilibrium Binding Analysis of the Progesterone Receptor. Equilibrium binding analysis was performed as described by Theofan and Notides (1984) with the exception that a 20-fold molar excess of cortisol relative to the concentration of [^{3}H]progesterone was included in all incubations.

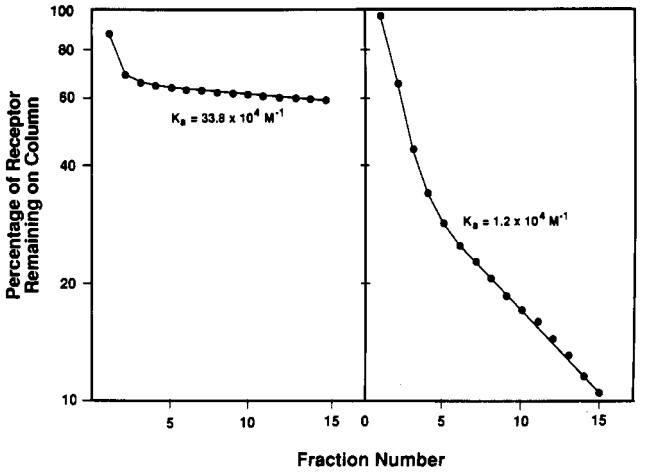

FIGURE 1: Elution of the estrogen and progesterone receptors from DNA–Sepharose. Partially purified estrogen (left) and progesterone (right) receptors were prepared from calf uteri. The receptors were incubated with saturating levels of [^{3}H]steroid and chromatographed in TDE buffer containing 180 mM KCl as described under Methods. Binding constants were determined from the slope of each elution profile from fraction 5 through fraction 15.

Coupling of DNA to Sepharose. Calf thymus DNA was covalently linked to Sepharose 4B by the method of Arndt-Jovin et al. (1975). The amount of bound DNA was measured by digesting the DNA from DNA–Sepharose with DNase I and measuring the amount of phosphate by the method of Chen et al. (1955) as modified by Arndt-Jovin et al. (1975).

Data Analysis. The calculations for the affinity of the receptors to DNA, the number of ions released upon binding of the receptors to DNA, and the Scatchard (1949) and Hill (1910) analysis of the binding of progesterone to its receptor were carried out by using Lotus 1-2-3. Linear regression via Lotus 1-2-3 yields the slope of the line, the value of r^2, the y-intercept, and the standard error of the slope and intercept. Correlation coefficients (r^2) of the Hill plots were 0.95 and greater. Correlation coefficients (r^2) of the region of the transformed elution profile from which K_a, for DNA, was measured were 0.98 and greater. Analysis of the concentration dependence of the Hill coefficient was carried out by nonlinear regression using the Enzfitter data analysis program. The program calculated the values of four parameters to best fit the data. These parameters are the limiting values of the Hill coefficients at high and low receptor concentrations, the dimerization constant, and a parameter that describes the steepness of the transition from a state in which the Hill coefficient is the limiting value at low receptor concentrations to the state in which the Hill coefficient is the limiting value at high receptor concentrations. All results are expressed as the mean ± SE.

RESULTS

Affinity of Estrogen and Progesterone Receptors for DNA. The affinity of the estrogen and progesterone receptors for DNA was measured by using isocratic elution from DNA–Sepharose. The elution profiles of each receptor exhibited an initial steep slope, followed by a less steep portion (fractions 5–15) from which the affinity for DNA was determined (Figure 1). The initial steep portion of the elution profile was due to receptor which did not bind to DNA. When Sepharose lacking DNA or buffer containing 1 M KCl was used, over 90% of the receptor was eluted as the "steep" component (not shown). Purification of the estrogen receptor decreases the proportion of receptor eluting in the steep component, but has no effect on the actual affinity of the receptor for DNA (Bond & Notides, 1987). Gel filtration of cytosol, which increases the proportion of the estrogen receptor which interacts with DNA (formerly termed "activation"), also decreases the proportion of receptor eluting in the initial component but has no effect on the affinity of the receptor for DNA (Skafar & Notides, 1987).

Table I: Affinity of the Estrogen and Progesterone Receptors for DNA[a]

estrogen receptor		progesterone receptor	
	$K_a \times 10^4$ (M^{-1})		$K_a \times 10^4$ (M^{-1})
estradiol bound	29.5 ± 2.1	progesterone bound	1.2 ± 0.1
hormone free	15.9 ± 0.7	hormone free	1.4 ± 0.2
high concentration	24.9 ± 2.5		
low concentration	7.9 ± 0.4		

[a] The affinity of each receptor for calf thymus DNA was first measured at 180 mM KCl and 6–12 nM receptor. The results shown are averages of two independent experiments (first two rows of the table). The affinity of the estrogen receptor for DNA was then measured at 180 mM KCl by using either high (>6 nM) or low (<3 nM) concentrations of receptor, in the presence of [^{3}H]estradiol. The results shown are averages ± SEM of three independent experiments (last two rows of the table).

The affinity of the estrogen and progesterone receptors for DNA was first investigated at receptor concentrations of 6 nM and greater. At these concentrations, the estrogen receptor exhibits a hormone-dependent increase in its affinity for DNA (Skafar & Notides, 1985). The affinity of the hormone-free estrogen receptor for DNA was over 10-fold higher than the affinity of the hormone-free progesterone receptor for DNA (K_a = 15.9 × 10^4 M^{-1} vs 1.4 × 10^4 M^{-1}, respectively) (Figure 1; Table I). A second difference between the two proteins was in the effect of hormone binding. Binding estradiol to its receptor increased the receptor's affinity for DNA from 15.9 × 10^4 M^{-1} to 29.5 × 10^4 M^{-1} (Table I) whereas binding progesterone to its receptor had no effect on the affinity of the receptor for DNA (1.2 × 10^4 M^{-1} vs 1.4 × 10^4 M^{-1}; Table I).

The 10-fold difference between the affinities of the estrogen and progesterone receptors for DNA and the lack of effect of progesterone binding to its receptor were not expected due to the homology between the two proteins. Since reducing the concentration of the estrogen receptor to values of 3 nM and less decreases its affinity for DNA and eliminates the effect of hormone binding on the receptor's affinity for DNA (Skafar & Notides, 1985, 1987; Yamamoto & Alberts, 1974), the affinity of the estrogen receptor for DNA was measured at low receptor concentrations to confirm the earlier results. Reducing the concentration of the receptor decreased its affinity for DNA to one-third its value at high receptor concentrations (Table I). This was not due to changing the total concentration of protein, as diluting the receptor with an aliquot of the resuspended ammonium sulfate precipitate that had been briefly heated to inactivate the estrogen receptor had no effect on the affinity for DNA (not shown). The diluted estrogen receptor's affinity for DNA was still greater than the progesterone receptor's by about 5-fold. Reducing the concentration of progesterone receptor had no effect on its affinity for DNA (not shown).

Determination of the Number of Ions Released upon Binding Each Receptor to DNA. The number of ions released when each receptor bound DNA was determined (Record et al., 1976) to investigate the conformation of each receptor. No difference between the affinity of the hormone-free or hormone-bound progesterone receptor for DNA was detected at any salt concentration (Table II; Figure 2). The number

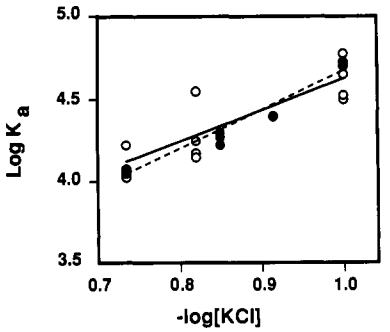

FIGURE 2: Salt dependence of the affinity of the progesterone receptor for DNA. The affinity of the progesterone receptor for DNA was measured in the absence (O) or presence (●) of [^{3}H]progesterone in TDE buffer containing 100–180 mM KCl. Receptor concentrations were between 6 and 12 nM.

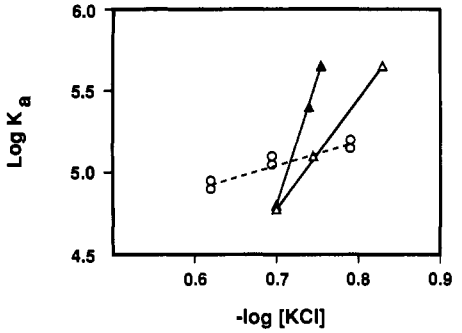

FIGURE 3: Salt dependence of the affinity of the estrogen receptor for DNA. The affinity of the estrogen receptor for DNA was measured in the presence (▲) or absence (△) of [^{3}H]estradiol at receptor concentrations of 6–10 nM. The affinity of the estrogen receptor for DNA was also measured at receptor concentrations between 2 and 3 nM, in the presence of [^{3}H]estradiol (O). Affinities were measured in TDE buffer containing 150–240 mM KCl.

Table II: Stoichiometry of Ion Release and Maximum Number of Ion Pairs (m') between the Steroid Receptors and DNA[a]

receptor, concn	ligand	ions released	max ion pairs (m')
estrogen >6 nM	estradiol	12.1 ± 0.8	13.8 ± 0.9
estrogen, >6 nM	none	6.7 ± 0.2	7.6 ± 0.2
estrogen, <3 nM	estradiol	1.8 ± 0.2	2.1 ± 0.2
progesterone, >6 nM	progesterone	2.6 ± 0.1	2.9 ± 0.1
progesterone, >6 nM	none	2.0 ± 0.3	2.3 ± 0.4

[a] The slopes (α) of the best-fit lines shown in Figures 2 and 3 directly yield the stoichiometry of ion release. The maximum number of ion pairs between each protein and DNA was then determined according to the equation $\alpha = 0.88m'$. The values of the maximum number of ion pairs were calculated on the assumption that no anion or proton release occurred.

of ions released was also the same: 2 ± 0.3 for the hormone-free receptor and 2.6 ± 0.1 for the hormone-bound receptor (Table II).

Hormone binding increased the number of ions released when the estrogen receptor bound DNA from 6.7 ± 0.2 to 12.1 ± 0.8 (Table II). Reducing the concentration of receptor decreased the number of ions released when the hormone-bound receptor bound DNA from 12.1 to 1.8 ± 0.2 (Figure 3; Table II). This value was similar to the number of ions released when the hormone-bound progesterone receptor bound DNA, 2.6 ± 0.1 (Table II).

Hormone-Binding Mechanism of the Progesterone Receptor. The calf uterine progesterone receptor has been previously shown to bind progesterone with a positive cooperative binding mechanism: the Scatchard plot is curved, and the Hill coefficient is 1.22 ± 0.2 (Theofan & Notides, 1984). Similar results are shown in Figure 4 for progesterone binding at high

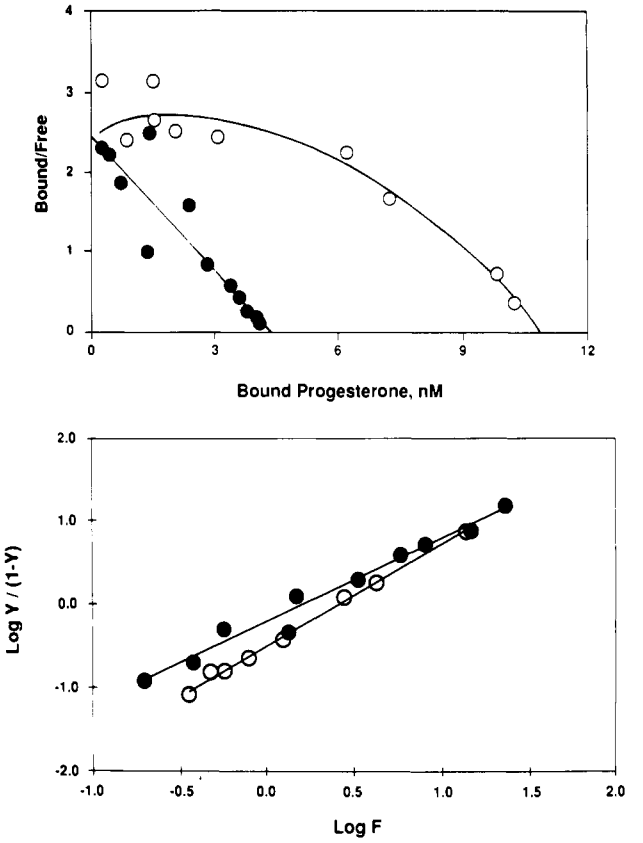

FIGURE 4: Equilibrium binding analysis of the binding of [^{3}H]-progesterone to the progesterone receptor. Equilibrium binding analysis was performed with calf uterine cytosol as described under Methods. Scatchard (top) and Hill (bottom) plots of representative experiments at receptor concentrations of 11.2 nM (O) and 4.3 nM (●) are shown. The corresponding Hill coefficients of 1.22 ± 0.06 and 1 ± 0.04, respectively, were determined by linear regression using Lotus 1-2-3.

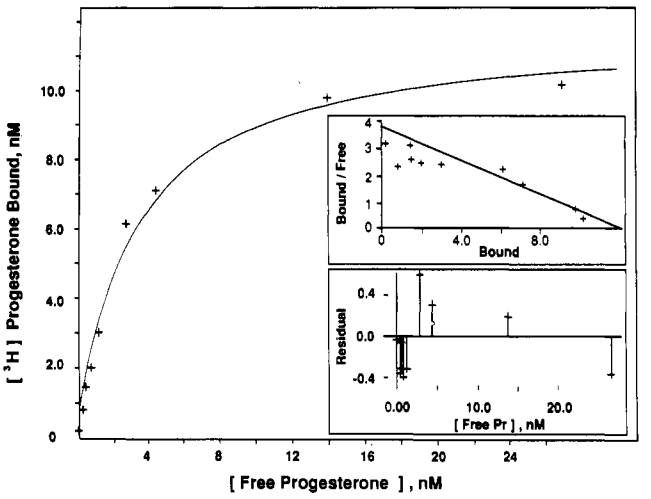

FIGURE 5: Fit of binding data to a noncooperative (hyperbolic) model. The specifically bound [^{3}H]progesterone (data of Figure 4, high concentration of receptor) was plotted vs the concentration of free [^{3}H]progesterone. The curve shown is the best fit determined by nonlinear regression analysis (Methods). (Lower inset) The difference between the actual and the calculated value of the specific binding was plotted vs the free progesterone concentration. (Upper inset) Scatchard plot of the binding data. The line shown is the linear isotherm obtained by using the values of B_{max} and K_d calculated from the nonlinear regression analysis of the original binding data.

receptor concentration: the Scatchard plot was curved, and the corresponding Hill coefficient obtained from the Hill plot was 1.22 ± 0.06. To more rigorously analyze the hormone-binding mechanism of the receptor, the saturation binding data

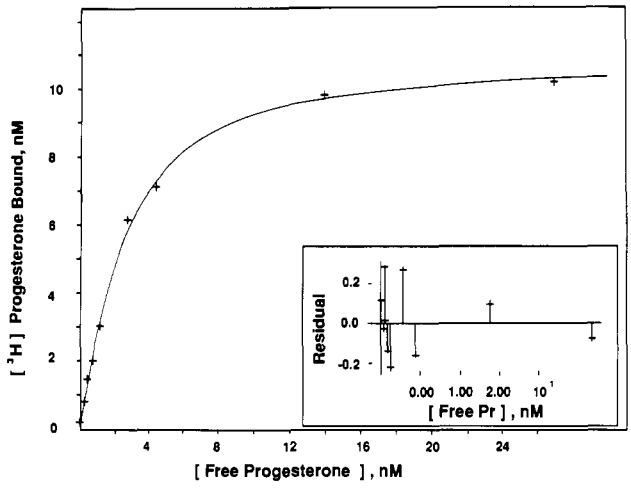

FIGURE 6: Fit of binding data to an allosteric (Hill) model. The binding data used in Figure 5 were fit to the Hill equation; the curve shown is the best fit determined by nonlinear regression analysis (Methods). (Inset) The difference between the actual and the calculated value of the specific binding was plotted vs the free progesterone concentration.

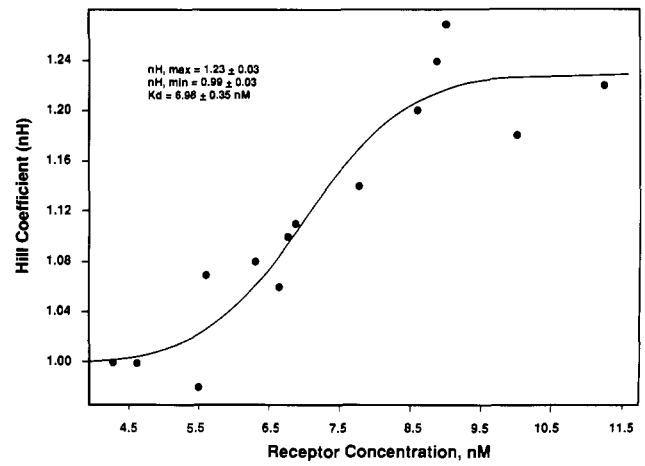

FIGURE 7: Dependence of the Hill coefficient on the concentration of progesterone receptor. Hill coefficients and receptor concentrations were determined by analyzing the equilibrium binding of [^{3}H]-progesterone to the cytosolic calf uterine progesterone receptor. Each point represents a separate binding analysis. The line shown is the best-fit curve determined by nonlinear regression as described under Methods.

were fit to a noncooperative, hyperbolic model (Figure 5) and an allosteric (Hill) model (Figure 6) by nonlinear regression.

When the data were fit to a noncooperative (hyperbolic) model, a reduced χ^2 of 0.14 was obtained (Figure 5). Also, the residuals of y vs x showed systematic deviations: the points at low concentrations of free progesterone were negative, the next three were positive, and the point at the highest free progesterone concentration was negative (Figure 5, lower inset). This is the pattern predicted to occur when binding data for an allosteric protein are fit to a simple hyperbolic binding isotherm. When the B_{max} and the K_d of binding calculated by nonlinear regression of the binding data were used to draw a linear isotherm on the Scatchard plot, systematic deviations of the line from the data were observed: the fit was close at high concentrations of bound progesterone, but not at low concentrations (Figure 5, upper inset).

When the same binding data were fit to an allosteric (Hill) model (Figure 6), a reduced χ^2 of 0.04 was obtained. The residuals of y vs x (Figure 6, inset) were both smaller and more evenly distributed than in Figure 5. Note the difference in scale of the y-axes of the residuals (Figures 5 and 6, insets). The calculated Hill coefficient was 1.27 ± 0.06. These analyses confirmed the earlier report (Theofan & Notides, 1984) which showed the binding of progesterone by the calf receptor is best described as positive cooperative.

Dimerization Constant of the Progesterone Receptor. The progesterone receptor exhibited DNA-binding properties similar to those of the monomeric estrogen receptor. The dimerization constant of the receptor was measured in order to determine whether the progesterone receptor could be monomeric at the concentrations of progesterone receptor used (6–12 nM).

The shift in the binding mechanism for progesterone from a positive cooperative interaction, as indicated by a Hill coefficient greater than 1, to a noncooperative mechanism as the receptor concentration decreased was used to measure the dimerization constant. For example, at a receptor concentration of 11.2 nM the Scatchard plot of progesterone binding was curved and the corresponding Hill coefficient was 1.22 ± 0.06 (Figure 4). At a receptor concentration of 4.3 nM, a linear Scatchard plot was obtained and the Hill coefficient was 1.0 ± 0.04 (Figure 4). The results of multiple equilibrium binding analyses using receptor concentrations between 4 and 12 nM are shown in Figure 7. The best-fit line shown (reduced χ^2 = 0.0012) was determined by nonlinear regression. The limiting value of the Hill coefficient at low concentrations of receptor was 0.99 ± 0.03. The limiting value of the Hill coefficient at high concentrations of receptor was 1.23 ± 0.03. This agreed closely with the Hill coefficient for progesterone binding, 1.21 ± 0.02, n = 4, obtained in an independent series of experiments using receptor concentrations of 7.7 nM and greater (not shown). From the data in Figure 7 the dimerization constant was determined to be ≈7 ± 0.4 nM.

Discussion

The hormone-free estrogen and progesterone receptors have a 10-fold difference in their affinity for DNA when measured at the same receptor concentration and 180 mM KCl. The difference in affinity most probably results from a difference in the oligomeric state of the two proteins.

The calf estrogen receptor undergoes changes in its biochemical properties as the receptor concentration is altered. At receptor concentrations of about 0.3 nM, a nearly linear Scatchard plot of [^{3}H]estradiol binding is obtained and the Hill coefficient is 1.1 (Notides et al., 1981). As the receptor concentration is increased to 1–2 nM, the Scatchard plot of [^{3}H]estradiol binding becomes more convex and the Hill coefficient increases to a maximum of 1.6 (Notides et al., 1981). Similarly, the proportion of receptor exhibiting a slow dissociation rate for [^{3}H]estradiol decreases and the proportion of receptor exhibiting a fast dissociation rate for [^{3}H]estradiol increases as the receptor concentration is decreased (Weichman & Notides, 1977, 1979; Skafar & Notides, 1987). Also, hormone binding increases the affinity of the receptor for DNA at receptor concentrations of 6 nM and greater, whereas at receptor concentrations less than 3 nM, hormone binding has no effect on DNA binding (Skafar & Notides, 1985). Finally, dilution of the receptor decreases the proportion of receptor sedimenting as the 5S dimeric species and increases the proportion sedimenting as the 4S monomeric species (Skafar & Notides, 1987). The dependence of allosteric effects, such as those observed on hormone and DNA binding, on subunit–subunit interactions within an oligomer is a characteristic of allosteric proteins (Monod et al., 1965; Koshland, 1970). That ≈12 ions were released when the hormone-bound receptor bound DNA at high receptor concentrations and only 2–3

when diluted is consistent with the idea that site–site interactions within the dimer influence the conformation of the individual subunits.

The number of ion pairs between the estrogen receptor and DNA calculated from the data reported here are in excellent agreement with earlier results (Skafar & Nitides, 1985, 1987). However, in this study the dimeric form of the estrogen receptor exhibited a 3-fold higher affinity for DNA than did the monomeric form. This is somewhat different than other results showing a 15-fold greater affinity of the dimeric estrogen receptor for DNA compared with that of the monomeric estrogen receptor (Yamamoto & Alberts, 1974). This difference may reflect differences between the bovine and rat estrogen receptors, the different salt concentrations and pH used in the two studies, or a combination of these factors.

Several observations indicate the progesterone receptor was in the monomeric form in the DNA-binding studies reported here. First, 2–3 ions were released when the progesterone receptor and the monomeric estrogen receptor bound DNA. This similarity is consistent with the extensive homology between the DNA-binding domains of the two proteins (Misrahi et al., 1987). Dilution of the progesterone receptor did not change its affinity for DNA. Second, progesterone had no effect on the binding of the progesterone receptor to DNA. The inability of progesterone binding to alter its receptor's affinity for DNA is similar to the behavior of the monomeric estrogen receptor. Third, the dimerization constant of the progesterone receptor, ≈7 nM, is over 10-fold greater than the dimerization constant of the estrogen receptor, ≈0.3 nM (Notides et al., 1981). This observation increases the probability that the two receptors exist in different states at the same concentration.

The maximum Hill coefficient for progesterone binding to the calf uterine receptor obtained in these experiments, 1.23 ± 0.03, agreed closely with earlier work (Theofan & Notides, 1984). The Hill coefficient decreased progressively with receptor concentration to a value of 0.99 ± 0.03. This agreed well with the theoretical value of 1 for a monomer or a noncooperative mechanism. The decrease was not due to changes in total protein concentration, but to alterations in receptor concentration. The comparatively high dimerization constant of the progesterone receptor, ≈7 nM, will have profound effects on its biochemical properties in vitro. It helps explain the few reports documenting cooperative binding of progesterone, since receptor concentrations greater than 8 nM were required to exhibit maximum cooperativity (Theofan & Notides, 1984). This result emphasizes the importance of specifying the receptor concentration used in biochemical studies.

The dimerization constant of the estrogen receptor, measured by the change in Hill coefficient with receptor concentration, is ≈0.3 nM (Notides et al., 1981). However, the estrogen receptor's affinity for DNA increases at receptor concentrations between 3 and 6 nM (Skafar & Notides, 1985). Similarly, at receptor concentrations greater than 8 nM the progesterone receptor exhibits its maximum Hill coefficient, which is indicative of the dimeric form of the receptor; when its affinity for DNA was measured at this same concentration, however, the protein behaved as a monomer. Several explanations for these discrepancies are possible. First, the large DNA excess used in the isocratic elution technique could actually promote the dissociation of the receptor to monomers (see the supplementary material). Second, about 60% of the receptor applied to a column binds to it. Since receptor concentrations are expressed as total receptor recovered from the column, the actual concentration is less than given. Finally, the continuous elution of the receptor from the column and the resulting decrease in receptor concentration may allow the dimeric receptor to dissociate during chromatography. This explanation is less likely than the others, since a linear elution isotherm, indicative of a single binding constant, was observed over the range from which binding constants were calculated (Figure 1). It is not possible from the data in this paper to unequivocally choose between these alternatives. Moreover, they are not mutually exclusive. These observations, however, do suggest that comparing dimerization constants in the absence of DNA, in the presence of DNA, and in the presence of specific hormone response elements to examine the relation between DNA concentration and receptor dimerization may result in further insights into receptor structure.

The 10-fold difference in dimerization constants between the estrogen and progesterone receptors could have important consequences for steroid hormone action in vivo. Since the receptor dimer is the species that binds to the hormone response element in vivo (Tsai et al., 1988), a change in its concentration could influence the sensitivity and responsiveness of a cell to hormone. Receptor dimerization has also been recently implicated in the process of nuclear localization of the progesterone receptor (Guiochon-Mantel et al., 1989). The chicken oviduct exhibits seasonal variations in progesterone receptor concentration and response to hormonal stimulation (Spelsberg et al., 1979a,b; Seaver et al., 1980). In the human female, the uterine progesterone receptor concentration varies 4-fold during the menstrual cycle (Bayard et al., 1978). It is tempting to speculate a change in sensitivity to progesterone may also occur.

The dimerization constant of the estrogen receptor, ≈0.3 nM, is so low compared with the physiological receptor concentration of at least 40 nM (Sasson & Notides, 1983) that it most probably is in the dimeric form. In contrast, the dimerization constant of the progesterone receptor, ≈7 nM, is relatively high compared with the physiological concentration of 40 nM or more (Theofan & Notides, 1984), so that a larger proportion of the progesterone receptor could exist in the monomeric state. Detailed calculations of the proportion of monomer and dimer of each receptor in vivo are not possible because of uncertainty in the estimation of cellular receptor concentrations and because the effects of DNA concentration (see the supplementary material), hormone binding, and receptor phosphorylation (Dougherty et al., 1982; Horwitz et al., 1985; Logeat et al., 1985; Garcia et al., 1986; Migliaccio et al., 1986; Sheridan et al., 1988) on dimerization have not been quantified.

Measuring the DNA-binding properties of the dimeric calf uterine progesterone receptor by isocratic elution requires purification of receptor to obtain higher concentrations. The highly purified chicken progesterone receptor makes 7.7 ion pairs with DNA; its affinity for nonspecific DNA is slightly decreased, and its affinity for specific DNA is slightly increased, by steroid binding (Rodriguez et al., 1989). Steroid binding also slightly decreases the affinity of the rat glucocorticoid and rabbit progesterone receptors (Schauer et al., 1989) for DNA. It will be interesting to determine whether the calf receptor behaves similarly.

Comparing the properties of the calf uterine estrogen and progesterone receptors reveals that differences in the dimerization constant of the receptors are directly related to apparent differences in their affinity for DNA. Further quantitative studies of these receptors will lead to understanding how the structure of each steroid receptor is related to its mechanism of regulating gene expression.

ACKNOWLEDGMENTS

Many thanks to Dr. Angelo C. Notides and Dr. Dan Walz for their help and support and to Dr. Tom Taggart for his careful reading of the manuscript.

SUPPLEMENTARY MATERIAL AVAILABLE

An appendix, "Monomer–dimer equilibria in the presence of DNA", by Jeffrey L. Bond and Debra F. Skafar, presenting modeling studies which suggest that the presence of DNA can affect the monomer–dimer equilibrium of a DNA-binding protein and that the effect depends on the quaternary structure of the protein's DNA-binding site, including two figures (8 pages). Ordering information is given on any current masthead page.

Registry No. Progesterone, 57-83-0; estradiol, 50-28-2.

REFERENCES

Arndt-Jovin, D. J., Jovin, T. M., Bahr, W., Frischauf, A.-M., & Marquardt, M. (1975) *Eur. J. Biochem. 54*, 411–418.

Bayard, F., Damilano, S., Robel, P., & Baulieu, E.-E. (1978) *J. Clin. Endocrinol. Metab. 46*, 635–648.

Beato, M. (1989) *Cell 56*, 335–344.

Beato, M., Chalepakis, G., Schauer, M., & Slater, E. P. (1989) *J. Steroid Biochem. 32*, 737–748.

Bond, J. P., & Notides, A. C. (1987) *Anal. Biochem. 163*, 385–390.

Chen, P. S., Toribara, T. Y., & Warner, H. (1985) *Anal. Biochem. 28*, 1756–1758.

deHaseth, P. L., Gross, C. A., Burgess, R. R., & Record, M. T., Jr. (1977) *Biochemistry 16*, 4777–4782.

Denner, L. A., Bingman, W. E., Greene, G. L., & Weigel, N. L. (1987) *J. Steroid Biochem. 27*, 235–243.

Dougherty, J. J., Puri, R. K., & Toft, D. O. (1982) *J. Biol. Chem. 257*, 14226–14230.

Evans, R. M. (1988) *Science 240*, 889–895.

Garcia, T., Jung-Testas, I., & Baulieu, E.-E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7573–7577.

Guiochon-Mantel, A., Loosfelt, H., Lescop, P., Sar, S., Atger, M., Perrot-Applanat, M., & Milgrom, E. (1989) *Cell 57*, 1147–1154.

Hill, A. V. (1910) *J. Physiol. (Lond.) 40*, iv–vii.

Horwitz, F., Francis, M., & Wei, L. (1985) *DNA 4*, 45–46.

Koshland, D. E., Jr. (1970) in *The Enzymes* (Boyer, P. D., Ed.) pp 315–387, Academic Press, New York.

Kumar, V., & Chambon, P. (1988) *Cell 55*, 145–156.

Logeat, F., LeCunff, M., Pamphile, R., & Milgrom, E. (1985) *Biochem. Biophys. Res. Commun. 131*, 421–427.

McGhee, J. D., & von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469–489.

Migliaccio, A., Rotondi, A., & Auricchio, F. (1986) *EMBO J. 5*, 2867–2872.

Miller, J., McLachlan, A. D., & Klug, A. (1985) *EMBO J. 4*, 1609–1614.

Misrahi, M., Atger, M., d'Auriol, L., Loosfelt, H., Meriel, C., Fridlansky, F., Guiochon-Mantel, A., Galibert, F., & Milgrom, E. (1987) *Biochem. Biophys. Res. Commun. 143*, 740–748.

Monod, J., Wyman, J., & Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88–118.

Notides, A. C., Lerner, N., & Hamilton, D. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4926–4930.

Notides, A. C., Sasson, S., & Callison, S. (1985) in *Molecular Mechanism of Steroid Hormone Action* (Moudgil, V. K., Ed.) pp 173–197, De Gruyter, Berlin.

Record, M. T., Jr., Lohman, T. M., & deHaseth, P. (1976) *J. Mol. Biol. 107*, 145–158.

Rodriguez, R., Carson, M. A., Weigel, N. L., O'Malley, B. W., & Schrader, W. T. (1989) *Mol. Endrocrinol. 3*, 356–362.

Sasson, S., & Notides, A. C. (1983) *J. Biol. Chem. 258*, 8113–8117.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.

Schauer, M., Chalepakis, G., Willmann, T., & Beato, M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1123–1127.

Seaver, S. S., Van Eys, D. C., Hoffmann, J. F., & Coulson, P. B. (1980) *Biochemistry 19*, 1410–1416.

Sheridan, P. L., Krett, N. L., Gordon, J. A., & Horwitz, K. B. (1988) *Mol. Endocrinol. 2*, 1329–1342.

Skafar, D. F., & Notides, A. C. (1985) *J. Biol. Chem. 260*, 12208–12213.

Skafar, D. F., & Notides, A. C. (1987) in *Biochemical Actions of Hormones* (Litwack, G., Ed.) Vol. 14, pp 318–348.

Spelsberg, T. C., Boyd, P. A., & Halberg, F. (1979a) *Adv. Exp. Med. Biol. 117*, 225–268.

Spelsberg, T. C., Thrall, C., Martin-Dani, G., Webster, R. A., & Boyd, P. A. (1979b) in *Ontogeny of Receptor and Reproductive Hormone Action* (Hamilton, T. H., & Clark, J. H., Eds.) pp 31–63, Raven Press, New York.

Theofan, G., & Notides, A. C. (1984) *Endocrinology 114*, 1173–1179.

Tsai, S. Y., Carlstedt-Duke, J., Weigel, N. L., Dahlman, K., Gustafsson, J.-A., Tsai, M.-J., and O'Malley, B. W. (1988) *Cell 55*, 361–369.

Weichman, B. M., & Notides, A. C. (1977) *J. Biol. Chem. 252*, 8856–8862.

Weichman, B. M., & Notides, A. C. (1979) *Biochemistry 18*, 220–225.

Yamamoto, K. R., & Alberts, B. (1974) *J. Biol. Chem. 249*, 7076–7086.