Increase in the Stability and Helical Content of Estrogen Receptor α in the Presence of the Estrogen Response Element: Analysis by Circular Dichroism Spectroscopy†

Norma Greenfield,‡ Veena Vijayanathan,§ T. J. Thomas,§ Michael A. Gallo,|| and Thresia Thomas*,||

Departments of Neuroscience and Cell Biology, Medicine, and Environmental and Community Medicine, Environmental and Occupational Health Sciences Institute, and The Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

Received December 15, 2000; Revised Manuscript Received March 29, 2001

ABSTRACT: Ligand-dependent stabilization of the estrogen receptor (ER) is often postulated, with limited support from experimental data. We studied the thermal unfolding of recombinant ERα by circular dichroism (CD) spectroscopy. The T_M of unfolding of ERα was 38 ± 2.4 °C, and the van't Hoff enthalpy of unfolding was 31.7 ± 3.4 kcal/mol in the absence of ligands. Addition of estradiol (E_2) increased the T_M to 43.6 ± 2.3 °C, while addition of E_2 and an oligonucleotide harboring the estrogen response element (ERE) increased the T_M to 47.9 ± 1.6 °C. Addition of the antiestrogen 4-hydroxytamoxifen (HT) alone did not increase the T_M; however, a combination of HT and the ERE increased the T_M to 48.9 ± 1.0 °C. The ERE alone increased the T_M to 46.1 ± 0.9 °C. Addition of E_2 alone had no effect on the apparent enthalpy of unfolding; however, the ERE alone increased the apparent enthalpy from 31.7 to 36.1 kcal/mol. ERα samples containing the ERE also exhibited an increase in the negative ellipticity at 208 and 222 nm, relative to that of ligand-free ERα, suggesting a stabilization of the α-helix. CD data analysis further showed that the presence of the ERE caused a large increase in α-helical content of ERα in both the presence and absence of the ligands. This increase in α-helical content of ERα was not observed in the presence of a nonspecific oligonucleotide. These results show that the ERE can increase the thermal stability of ERα, enhance its α-helical content, and facilitate the cooperativity of the folding transition.

Estrogen receptors (ERα and ERβ)[1] are ligand-activated transcription factors that mediate the actions of the female sex hormone, estradiol, its metabolites, and synthetic mimics (*1*−*4*). Estradiol and other ligands are believed to alter the conformation of ER so that it acquires a high affinity for DNA and binds to specific sequences, the ERE, present in the promoter/enhancer region of estrogen-responsive genes. This binding and the associated changes in the chromatin structure initiate transcriptional activation of estrogen-responsive genes. Activation of a cell- and tissue-specific network of genes enables estradiol and ERs to exert multiple functions, not only on the reproductive system but also on several other organ systems (*5*, *6*).

Recent X-ray crystallographic studies on the ligand binding domain of ER (α and β) have identified the positioning of the ligand in a well-defined hydrophobic pocket of the protein (*7*−*9*). Similar studies on the DNA binding domain have indicated that the protein binds as a symmetrical dimer to the palindromic consensus sequence (*10*, *11*). There has been no crystallographic or NMR study on the whole protein to assess ligand-induced changes in the total ER protein structure.

A conventional avenue for exploring ligand-induced conformational changes has been to examine changes in protease sensitivity due to ligand or DNA binding. Studies on protease sensitivity of the whole protein have produced contradictory results. Fritch et al. (*12*) did not observe any difference in the proteolytic pattern of unbound ER, or estrogen/antiestrogen-bound ER. Emmus et al. (*13*) also did not find any difference in the sensitivity of ER bound to estrogens or antiestrogens to trypsin, chymotrypsin, papain, or elastase. However, Attardi and Happe (*14*) reported differences in the sensitivity to proteolysis of the nuclear receptor bound to estrogen and antiestrogens. More recent studies by Kraichely et al. (*15*) demonstrated distinct differences in the protease digestion pattern of ER (α and β) bound to agonistic or antagonistic ligands. Studies on the DNA binding domain of ERα also showed differences in chymotrypsin sensitivity when bound to classical and altered EREs (*16*). However, direct measurements of ligand-induced conformational changes in intact ER have not been possible.

† This work was supported by Public Health Service Grants CA42439, CA73058, and CA80163 from the National Cancer Institute and Grant ES05022 from the National Institute of Environmental Health Sciences.

* To whom correspondence should be addressed: 125 Paterson St., Clinical Academic Building, Room 7092, New Brunswick, NJ 08903. Phone: (732) 235-8458. Fax: (732) 235-8473. E-mail: thomasth@umdnj.edu.

‡ Department of Neuroscience and Cell Biology.

§ Department of Medicine.

|| Department of Environmental and Community Medicine.

[1] Abbreviations: ER, estrogen receptor; CD, circular dichroism; ERE, estrogen response element; E_2, estradiol; HT, 4-hydroxytamoxifen; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; ODN, oligodeoxyribonucleotide; DTT, dithiothreitol; DCC, dextran-coated charcoal; EMSA, electrophoretic mobility shift assay; TEGD, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, and 10% glycerol.

10.1021/bi002846l CCC: $20.00
Published on Web 05/10/2001

CD spectral measurements (*17*) of recombinant ERα provide a sensitive technique for examining the interactions between the ligand−receptor complex and the ERE. In the study presented here, we determined the helical contents of recombinant ERα using CD spectroscopy in the presence and absence of E_2, HT (the active metabolite of the antiestrogen tamoxifen), and an oligodeoxyribonucleotide harboring the consensus ERE. Spectral changes indicated that the agonist and antagonist provoked changes in the conformation of ERα, with the presence of the ERE inducing the largest increase in α-helical content. Thermal unfolding studies of ERα using CD spectroscopy further showed a significant stabilization of ERα protein structure in the presence of the ERE.

MATERIALS AND METHODS

Materials. [^{3}H]E_2 was from New England Nuclear (NEN) Life Science Products, Inc. (Boston, MA). The specific activity of [^{3}H]E_2 was 72 Ci/mmol. Full-length recombinant human ERα was purchased from Panvera Corp. (Madison, WI). The stock solution was in 50 mM Tris-HCl (pH 8), 500 mM KCl, 2 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM sodium orthovanadate, and 10% glycerol. The dextran-coated charcoal (DCC) assay for ERα ligand binding activity yielded a concentration of 1040 ± 140 pmol/mL or ∼66 μg/mL.

DCC Assay and Sucrose Density Gradient Analysis of ERα. For these assays, an ERα stock solution was diluted 1000-fold in a buffer containing 10 mM Tris, 1 mM EDTA, 2 mM dithiothreitol, 10% glycerol, and 1 mg/mL BSA. ERα was incubated with 5 nM [^{3}H]E_2 for 3 h at 4 °C (*18*, *19*). This concentration was determined to be at the saturation level by Scatchard analysis. After incubation, unbound E_2 was removed by incubating it with dextran-coated charcoal (final concentrations, 0.01% dextran and 0.1% charcoal) for 10 min at 4 °C. The samples were then centrifuged, and the amount of radioactivity of the supernatant was determined by liquid scintillation counting. The level of nonspecific binding was determined in parallel samples incubated with 5 nM [^{3}H]E_2 alone or in combination with a 100-fold molar excess of unlabeled E_2. Concentrations of ERα were determined from the specifically bound [^{3}H]E_2. For the sucrose density gradient assay, the supernatant was loaded on a 10 to 30% linear sucrose gradient in TEDG buffer. Gradients were centrifuged in a Beckman SW60 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 250000*g* for 16 h (*18*, *19*). Fractions were collected into 5 mL of scintillation fluid, and the amount of bound radioactivity was quantified using a scintillation counter.

Polyacrylamide Gel Electrophoresis. Recombinant ERα was analyzed by SDS−polyacrylamide gel electrophoresis. Protein (0.5, 1, 2.5, and 5 μg) was diluted with a buffer containing 125 mM Tris-HCl (pH 8.0), 4% SDS, 30% glycerol, and 150 mM dithiothreitol. The samples were boiled for 5 min, loaded on a 10% polyacrylamide gel, and electrophoresed at 200 V in a running buffer (192 mM glycine, 25 mM Tris base, and 0.1% SDS). The gel was fixed and stained with a Coomassie brilliant blue solution (0.25% Coomassie brilliant blue in 45% methanol and 10% glacial acetic acid) for 3 h and destained in water for 4 h. Protein molecular mass markers (NEN Life Science Products, Inc.) were included. The markers consisted of *Escherichia coli* β-galactosidase (116 kDa), bovine serum albumin (66 kDa), chicken egg ovalbumin (45 kDa), and bovine erythrocyte carbonic anhydrase (29 kDa).

Oligodeoxyribonucleotides. HPLC/PAGE-purified ODNs were purchased from Oligos, Etc., Inc. (Wilsonville, OR). The integrity of the ODNs was tested by 5′-end labeling, followed by polyacrylamide gel electrophoresis. A 41-nucleotide ODN containing the consensus ERE and a 38-nucleotide complementary ODN was used for EMSA (*20*−*22*). A mutant ERE with two base pair difference was also used for EMSA. ODNs were dissolved in a buffer containing 10 mM Tris-HCl (pH 7.5) and 50 mM NaCl, and dialyzed three times against the same buffer before being used. An ODN having a similar distribution of purines and pyrimidines and GC content, arranged in a scrambled sequence, was used as a control ODN. Base sequences of the ERE, mutant, and control ODNs are listed with the palindromic ERE and corresponding mutant sequence underlined: ERE, 5′-GATC-CA<u>GGTCA</u>GAG<u>TGACC</u>TGAGCTAAAATAA-CACATTCAG-3′ and 3′-GGTCCAGTCTCACTGGACTC-GATTTTATTGTGTAAGT-5′; mutant ERE, 5′-GATCCA-<u>GGTCA</u>GAG<u>TGCAC</u>TGAGCTAAAATAACACATTCAG-3′ and 3′-CTAGGTCCAGTCTCACGTGACTCGATTT-TATTGTGTAAGTC-5′; and control ODN, 5′-AAAGCTC-GCTTCCTGAAGACGTTCTCGAAGAGAAATCTCTT-3′ and 3′-CGAGCGAAGGACTTCTGCAAGAGCTTCT-CTTTAGAGAA-5′.

Electrophoretic Mobility Shift Assay. EMSA experiments were conducted using recombinant ERα, as previously described (*20*). The duplex ERE was labeled with [γ-^{32}P]-ATP using a 5′-end labeling kit from Boehringer Mannheim (Indianapolis, IN). Approximately 20000−30000 cpm of the ^{32}P-labeled probe was mixed with 500 ng of human recombinant ERα. The final concentrations in the binding buffer were 10 mM Tris-HCl, 150 mM KCl, 10% glycerol, 1 mM dithiothreitol, and 10 μg/mL poly(dI-dC)·poly(dI-dC). The binding reaction was allowed to proceed for 1 h at 4 °C, and for 30 min at 25 °C, and then the mixture loaded on a 6% polyacrylamide gel. Electrophoresis was performed at 200 V for 3 h. The gel was dried and exposed to Kodak Biomax MR-1 film for autoradiography for 24−48 h. The intensity of the DNA−protein band was quantified using a Scanjet 4c flatbed scanner (Hewlett-Packard), and analyzed using NIH Image version 1.6 software.

CD Spectroscopy. The stock solution of ERα (see above) was diluted 1:4 for the CD measurements. The final concentrations in the buffer were 20 mM Tris-HCl (pH 8), 125 mM KCl, 2 mM DTT, 0.25 mM EDTA, 0.25 mM sodium orthovanadate, and 2.5% glycerol. Data were collected on an Aviv model 64D circular dichroism spectrometer fitted with a five-compartment thermal equilibration chamber. Spectra were collected from 260 to 200 nm at 0.25 nm intervals, collecting for 2 s at each point. Data were smoothed using the method of Savitsky and Golay (*23*) and corrected for the contribution of the cells. Spectra were also corrected for the contribution of the ERE in samples containing ERα and the ERE.

Estimates of Secondary Structure Content. The secondary structure content of ERα in the absence and presence of estrogens, antiestrogens, and the ERE was estimated by fitting the data using the neural net program of Bohm et al.

(*24*) and by a nonconstrained least-squares fit of the data to peptide reference standards, as described by Brahms and Brahms (*25*).

Thermal Stability Measurements. To measure the effect of the ligands on the thermal stability of ERα, the ellipticity at 222 nm was followed as a function of temperature. Points were taken from 25 to 70 °C at 0.2 °C intervals, collecting data at each point for 5−10 s. To determine the apparent T_M and enthalpy of folding, data were fit to the following equations, assuming that all the ellipticity change was due to a two-state transition between the folded and unfolded receptor:

$$k = \exp\{[\Delta H/(RT)][(T/T_{Mobs}) - 1]\} \quad (1)$$

$$y = k/(1 + k) \quad (2)$$

$$\theta_{obs} = (\theta_{max} - \theta_{min})y + \theta_{min} \quad (3)$$

where θ_{obs} is the ellipticity found at any temperature, θ_{max} is the maximum ellipticity corresponding to fully folded ERα, θ_{min} is the ellipticity corresponding to the unfolded ERα, ΔH is the apparent van't Hoff enthalpy of folding, T_{Mobs} is the midpoint of the folding transition, and R is the gas constant (1.986 cal/mol). θ_{max}, θ_{min}, T_{Mobs}, and ΔH were estimated by nonlinear least-squares curve fitting using the commercial program SigmaPlot 2.0 (Jandel Scientific) (*25*, *26*). Note that the enthalpy calculated by this method is not a true thermodynamic parameter, because the unfolding was not reversible. In addition, no corrections were made to take into account the stoichiometry of the receptor−ligand complexes. The enthalpy measurements are given only as estimates of the cooperativity of the unfolding transition for comparative purposes.

RESULTS

Since the ligand binding activity of ERα is very labile, it is important to document the ligand binding activity and integrity of the protein before conducting the CD studies. Figure 1A shows a sucrose density gradient analysis of ERα. A stock solution of ERα was diluted 1000-fold in TEGD buffer and incubated with 5 nM [^{3}H]E_2 for 3 h, and the samples were analyzed with a 10 to 30% linear sucrose density gradient. The receptor sedimented as a 7S form, confirming the ligand binding to a discrete molecular form of the receptor. The sum of specifically bound radioactivity from the gradient was consistent with the level of binding determined from the DCC assay. Figure 1B shows the result of a SDS−polyacrylamide gel electrophoretic analysis of the ERα protein. Samples containing 0.5−5 μg of ERα were used for the electrophoretic analysis. A major band of the expected ERα protein was found at ~66000 Da, with a very light band of ~50000 Da in the lane loaded with 5 μg of the protein.

To confirm the DNA binding activity of ERα, EMSA was conducted. An oligonucleotide containing the ERE was end labeled with [γ-^{32}P]ATP and incubated with ERα. The reaction products were then analyzed by nondenaturing polyacrylamide gel electrophoresis, and autoradiography was conducted. Figure 2 shows the results of a typical EMSA showing the mobility of the free ERE and ERα·ERE complexes. A single band of the ERα·ERE complex was found even after extended autoradiographic exposure, suggesting that the lower-molecular mass protein does not have detectable ERE binding activity. Addition of unlabeled oligonucleotide consisting of a mutant ERE did not alter the intensity of the ERα·ERE complex, while addition of an

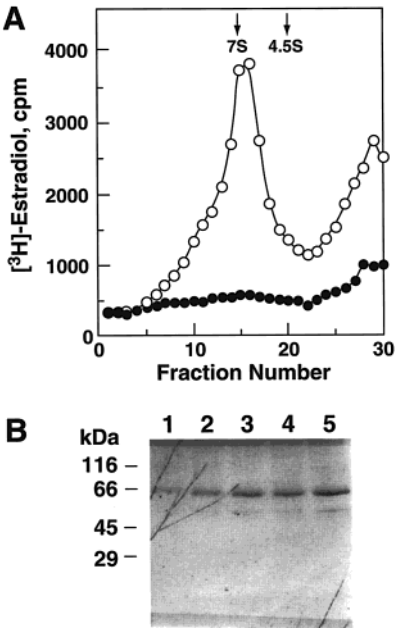

FIGURE 1: (A) Sucrose density gradient analysis of recombinant ERα. The stock solution was diluted 1000-fold and incubated with 5 nM [^{3}H]estradiol. The free ligand was removed by DCC treatment, and the supernatant was analyzed by a 10 to 30% linear sucrose gradient. Centrifugation was at 250000*g* for 16 h. Fractions were collected from the bottom of the gradient. ^{14}C-labeled γ-globulin (7S) and bovine serum albumin (4.5S) were used as external markers. Total binding (○) and nonspecific binding (●) in the presence of a 100-fold excess of unlabeled estradiol are shown. (B) SDS gel electrophoresis of recombinant ERα. Lanes contained 0.5, 1, 2.5, 2.5, and 5 μg of ERα protein. Protein bands were visualized by Coomassie brilliant blue staining.

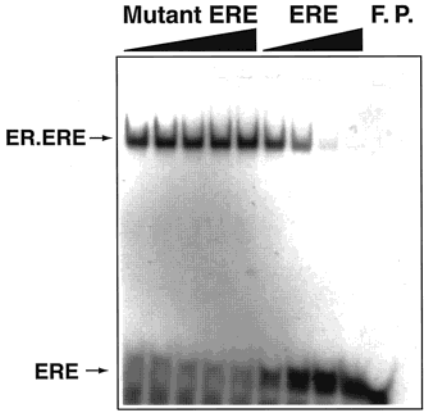

FIGURE 2: Electrophoretic mobility shift assay of recombinant ERα. Samples of ERα were incubated with ^{32}P-labeled ERE oligonucleotide in the binding buffer [10 mM Tris-HCl, 150 mM KCl, 10% glycerol, 1 mM dithiothreitol, and 10 μg/mL poly(dI-dC)·poly(dI-dC))], with increasing concentrations of an unlabeled mutant ERE or an unlabeled ERE. Protein DNA complexes were analyzed by native polyacrylamide gel electrophoresis. The first lane is control without the addition of unlabeled oligonucleotide. The following eight lanes contained samples in which an unlabeled mutant ERE or an unlabeled ERE was included at 5-, 10-, 50-, and 100-fold excess concentrations compared to the ^{32}P-labeled ERE. The last lane contained the ^{32}P-labeled ERE without ERα.

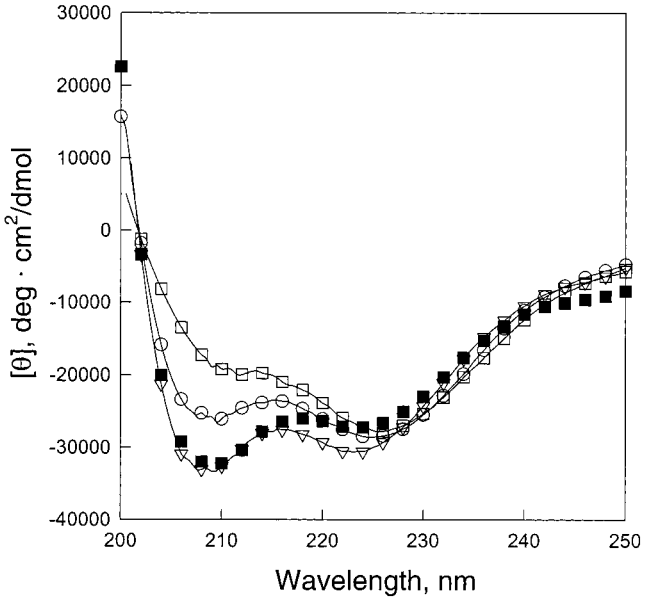

FIGURE 3: CD spectra of recombinant ERα in the presence of estradiol. Spectra from control (○), 5 μM estradiol (□), 5 μM ERE (■), and 5 μM estradiol and 5 μM ERE (▽) treatment groups are shown. The control sample received 1% ethanol, as an equivalent amount of ethanol was included in other samples as a solvent for estradiol. Spectral data are means from three separate experiments. Standard deviations were less than 10% of the mean.

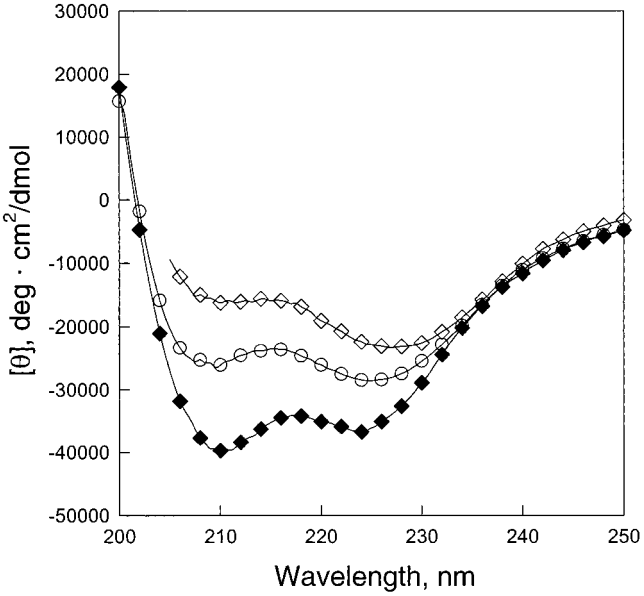

FIGURE 4: CD spectra of recombinant ERα in the presence of 4-hydroxytamoxifen (HT). Spectra from control (○), 5 μM HT (◇), and 5 μM HT and 5 μM ERE (◆) treatment groups are shown. The control sample received 1% ethanol. Spectral data are means from three experiments. Standard deviations were less than 10% of the mean.

unlabeled ERE competitively inhibited complex formation, demonstrating the specificity of the reaction. This result indicated that the recombinant ERα specifically bound to the ERE, and this binding could not be disrupted by the mutant oligonucleotide. We also found a supershift of the ERα·ERE complex in the presence of an anti-ERα antibody, indicating the ability of ERα to bind to an antibody specific to this protein (result not shown). Thus, the ERα used in our studies is functionally intact, as indicated by its ability to bind to [^{3}H]E_2, the ERE, and the anti-ERα antibody.

Figure 3 shows the effects of E_2, the ERE, and E_2 and the ERE on the CD spectrum of ERα. The CD data were analyzed using two different programs. The first, CDNN, is a neural net program that uses a protein database. The second, MLR, is a nonconstrained least-squares fit program that uses peptides in known conformations as standards, and does not utilize the protein concentration in its analysis. The MLR method is not usually as accurate as the CDNN method, but it was used in addition to the CDNN method because of the use of an indirect method for the determination of protein concentrations and the labile nature of the ligand binding activity of ERα. Both methods of analysis gave comparable results for the effect of estrogens and the ERE on the secondary spectrum of the protein. The results using the two methods are summarized in Table 1. Analysis of the spectra using either method suggests that the ligand-free ERα is 70−80% α-helical, with a very low β-sheet content of 2−3%, but 9−12% turns. Addition of E_2 caused a pronounced decrease in the ellipticity at 208 nm, and a small decrease at 222 nm, consistent with a small drop in helical content to ∼60% and an increase in β-turn and random coil content. Addition of the ERE, on the other hand, caused a large increase in the ellipticity at both 208 and 222 nm. Analysis of the spectra by either method suggested that the receptor became greater than 90% α-helical in the presence of the ERE. The conformational change caused by the addition of the ERE was almost the same in the absence or presence of E_2. However, addition of a nonspecific (control) oligonucleotide did not increase the α-helical content of the ERα protein (Table 1).

Figure 4 shows the effect of an antiestrogen, HT, as well as the ERE and HT on the CD spectrum of ERα. The trends in HT-induced effects on the ERα spectrum were very similar

Table 1: Effects of E_2, HT, and the ERE on the Secondary Structure of ERα[a]

	α		β		turn		random	
addition	CDNN	MLR	CDNN	MLR	CDNN	MLR	CDNN	MLR
none	0.81 ± 0.09	0.70 ± 0.08	0.04 ± 0.03	0.02 ± 0.04	0.09 ± 0.01	0.12 ± 0.03	0.10 ± 0.04	0.16 ± 0.16
5 μM E_2	0.7 ± 0.04	0.54 ± 0.1	0.03 ± 0.00	0.10 ± 0.14	0.06 ± 0.05	0.33 ± 0.19	0.11 ± 0.01	0.04 ± 0.05
5 μM HT	0.58 ± 0.05	0.48 ± 0.11	0.08 ± 0.01	0.03 ± 0.06	0.12 ± 0.01	0.31 ± 0.11	0.22 ± 0.04	0.18 ± 0.20
5 μM ERE	0.99 ± 0.00	0.91 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.09 ± 0.08
E_2 and ERE	0.97 ± 0.02	0.88 ± 0.03	0.01 ± 0.00	0.03 ± 0.05	0.06 ± 0.01	0.00 ± 0.00	0.02 ± 0.01	0.09 ± 0.08
HT and ERE	0.95 ± 0.01	0.86 ± 0.08	0.01 ± 0.00	0.00 ± 0.00	0.06 ± 0.00	0.00 ± 0.00	0.03 ± 0.01	0.14 ± 0.08
5 μM C-ERE		0.58 ± 0.08		0.28 ± 0.03		0.00 ± 0.00		0.20 ± 0.06
E_2 and C-ERE		0.62 ± 0.06		0.22 ± 0.01		0.00 ± 0.00		0.28 ± 0.17

[a] CDNN is the neural network analysis program of Bohm et al. (*24*), and MLR is a nonconstrained least-squares analysis procedure described by Brahms and Brahms (*25*). Data are the means ± standard deviation from three experiments. C-ERE refers to the control oligonucleotide.

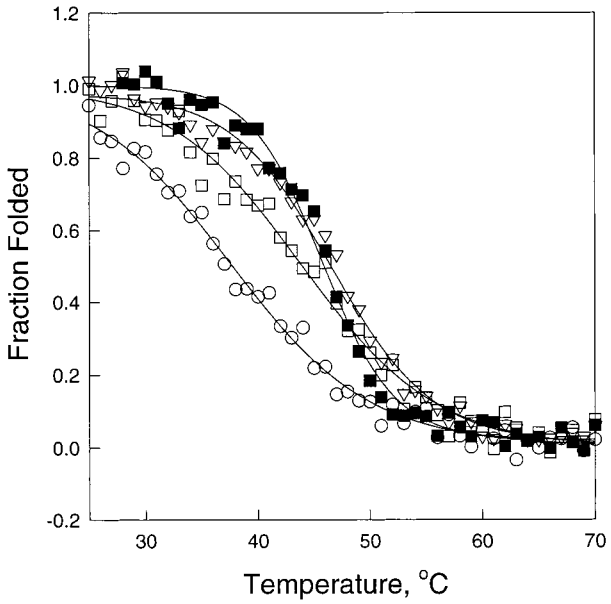

FIGURE 5: Unfolding profile of ERα to determine the T_M of unfolding. The ellipticity at 222 nm was monitored as a function of temperature, and the data were fit into a two-state model. Curves shown are control (○), 5 μM estradiol (□), 5 μM ERE (■), and 5 μM estradiol and 5 μM ERE (▽) treatment groups.

Table 2: Effect of E_2, HT, and the ERE on the Thermal Unfolding of ERα[a]

addition	apparent T_M (°C)	apparent enthalpy of unfolding (kcal/mol)
none (control)	38.0 ± 2.4	31.7 ± 3.4
5 μM E2	43.6 ± 2.3	31.7 ± 0.2
5 μM HT	38.4 ± 2.3	33.5 ± 1.9
5 μM ERE	46.1 ± 0.9	36.1 ± 3.1
E_2 and ERE	47.9 ± 1.6	48.3 ± 7.4
HT and ERE	48.9 ± 1.0	43.4 ± 1.1

[a] Data were fit to a two-state transition between a folded and unfolded state. The thermodynamic parameters are apparent because the unfolding was not reversible, and no corrections were made for the stoichiometry of the protein−ligand complexes. It was assumed that there was no heat capacity change during unfolding. Data are the means ± standard deviation from three experiments.

to those found with E_2, although there was a larger decrease in the α-helical content. Addition of the ERE in the presence of HT produced a large increase in α-helical content, similar to that of the E_2·ERα complex. Thus, our result shows evidence for conformational changes of ERα in the presence of an estrogen or an antiestrogen and the ERE. However, there were no large differences in the effect of an estrogen and an antiestrogen, especially in the presence of the ERE.

We next determined the stability of ERα under different conditions by measuring the ellipticity at 222 nm as a function of temperature. A representative unfolding profile of ERα in the presence E_2, the ERE, or E_2 and the ERE is presented in Figure 5. The analysis was performed by fitting the data to a two-state transition, as described in Materials and Methods. The T_M values and apparent enthalpies of the unfolding transition are presented in Table 2. The addition of E_2 to ERα caused a small but significant change in the T_M of unfolding ($P < 0.03$). In contrast, addition of HT had no effect on the T_M of folding of ERα (Table 2). Addition of the ERE to ERα in the absence of other ligands caused a significant increase in its stability ($P < 0.004$) and also increased the cooperativity of folding, as monitored by the increase in the apparent enthalpy of unfolding. When the ERE was added to ERα in the presence of either HT or E_2, there were additional changes. There was only a small increase in the T_M of unfolding in each case of ~2 °C, but there were significant changes in the cooperativity of unfolding, as indicated by the large increase in the apparent enthalpies of unfolding (Table 2). It is possible that the apparent increase in enthalpies of unfolding is due to a tighter binding of the ERE to ERα in the presence of E_2 or HT, which would increase the negative free energy of the folded state relative to the unfolded state.

DISCUSSION

Research on the ER has revolved around the concept that ligands, such as estradiol, induce conformational changes in the receptor so that it acquires the ability to bind to DNA. Originally, this concept was based on the observation that if cells or animals are treated with estradiol, the ER can be subsequently isolated from the cell nucleus, whereas in the untreated cells or animal tissues, the ER was found in the cytoplasmic fraction (*28*−*31*). Whether the ligand is required for the ER to induce a conformational state facilitating ERE binding remains equivocal as there are numerous studies demonstrating ER binding to the ERE in the absence of a ligand (*32*, *33*). While most of the biological effects of estradiol require the presence of the ER, it is unclear what stage of ER-induced transcriptional activation is dependent on the presence of the ligand. It is interesting to note that CD studies can distinguish the effects of the ligands on the ERα protein conformation from the thermal stability of the ERα protein. E_2 and HT produced similar changes in the overall conformational parameters, but only E_2 increased the thermal stability of ERα. On the other hand, the ERE produced changes in the conformational features and thermal stability of ERα, indicating that the ERE can be considered as an influential ligand of ERα.

The ER and other steroid receptors are known to exist as large complexes bound to the heat shock proteins in vivo (*28*−*31*). Recent studies have provided evidence for the existence of several families of coregulatory proteins that work with the steroid receptors in regulating transcription (*33*−*37*). The binding of the ligand disrupts ER binding to heat shock proteins and allows recruitment of coactivators in the case of estradiol and corepressors in the case of antiestrogens (*35*, *38*). A disruption of the unliganded ERα conformation by the addition of E_2 or HT was observed in CD studies as well. However, conformational changes induced by HT provide no advantage over the thermal stability of control ERα. This instability of HT-bound ERα may contribute to the inhibition of estrogenic effects by this anitestrogen.

The large increase in ellipticity at 222 nm and in thermal stability indicates that the ERα−ERE interaction is a clear example of DNA-induced conformational changes in the protein. ERE-induced conformational changes in ERα were previously indicated by changes in protease sensitivity of ERα in the presence of the ERE (*16*). ERα is also known to induce DNA bending in the ERE (*39*, *40*). Thus, the ERα−ERE interaction involves a mutually induced fit that increases the stability of the complex and thereby a mechanism for

screening out other closely related DNA sequences. This type of induced fit appears to be a common occurrence in the initiation of transcription, where poorly structured regions of the protein may form a regular secondary structure in the presence of DNA, enhancing protein−protein interactions and promoting an ordered nucleoprotein structure (*41*−*43*).

Addition of E_2 or HT leads to a conformational change as evidenced by a decrease in the negative ellipticity at 208 nm with a concomitant increase in the percentage of random coil (Figure 3 and Table 1). However, it is also possible that a change in tertiary structure, such as interactions between α-helices, such as in a coiled-coil α-helical protein, can also cause the CD spectrum of the α-helix to be perturbed (*44*). Interestingly, a functional and hydrophobicity cluster analysis of the N-terminal region of ERα indicates the requirement for an α-helical region in its function (*45*). In addition, DNA binding and ligand binding domains are known to have α-helical regions (*7*−*10*) which may interact in the presence of the ligand.

Estrogen- and antiestrogen-induced changes in the conformation of the ligand binding domain of the ER have been revealed in recent X-ray crystallographic studies (*7*−*9*). The main difference between the antiestrogen (raloxifene)-bound and E_2-bound ER was in the positioning of helix 12. The bulky side chain of raloxifene protrudes from the hydrophobic cavity formed from the alignment of multiple helices. When H12 is perfectly aligned over the cavity, as in the E_2·ER complex, this site becomes the prime site for interactions with coactivators. In the antiestrogen-bound ER, this site becomes the binding site for corepressor proteins (*9*, *46*). Thus, corepressor binding excludes coactivator binding and represses transcription. However, antiestrogens, such as HT, are also capable of acting as the agonist in the presence of certain coactivators, giving them "partial agonist" status (*34*, *47*). In this case, corepressor binding may be suppressed while the high affinity or flexibility of the coactivator becomes dominant. In the context of our studies, these and other data suggest that the major role of the ligand might be in the recruitment of coactivators and/or corepressors, not in the modulation of ER binding to the ERE. Indeed, the DNA binding domain of ERα alone is capable of binding to the ERE in EMSA experiments and has been crystallized with the ERE (*10*, *48*).

The ER is an important pharmacological target in breast cancer, since estradiol plays a large role in the origin and progression of this cancer (*49*, *50*). A subset of ER-positive breast tumors is completely dependent on the presence of estradiol for their continued growth (*51*). Under these conditions, depriving the tumor cells of estrogen or blocking its effect with tamoxifen produces breast tumor regression. However, tamoxifen is an agonist in the uterus, and therefore, long-term treatment with tamoxifen increases the risk of endometrial cancer (*51*, *52*). Extensive research is now targeted to synthesizing and/or characterizing selective ER modulators with desirable agonist or antagonist properties at different cellular contexts involving the expression of coactivators (*53*). CD studies of the ER in the presence of purified coactivator proteins may better distinguish the agonist and/or antagonist properties of novel ligands.

In summary, our results show increased thermal stability of ERα in the presence of the ERE. Distinct conformational changes, demonstrating an increased α-helical content of ERα, were found in the presence of the ERE. Estradiol also induced changes in the conformation and stability of ERα, although they were less remarkable than those induced by the ERE. There was a decrease in α-helical content of ERα in the presence of E_2 or HT, but conformational features of E_2·ER and HT·ER complexes were indistinguishable in the presence of the ERE. Thus, the effects of ligands on ERα function might be critical prior to ERE binding, perhaps at the stage of recruitment of coregulators. In the vicinity of the ERE, possible DNA-induced conformational changes in ERα may make the ligand's role less important to the dominant α-helical conformation of ERα.

REFERENCES

1. Greco, T. L., Duello, T. M., and Gorski, J. (1993) *Endocr. Rev. 14*, 59−71.
2. Tsai, M. J., and O'Malley, B. W. (1994) *Annu. Rev. Biochem. 63*, 451−486.
3. Jensen, E. V. (1996) *Ann. N.Y. Acad. Sci. 784*, 1−17.
4. Gustafsson, J. A. (1999) *J. Endocrinol. 163*, 379−383.
5. Green, P. S., and Simpkins, J. W. (2000) *Int. J. Dev. Neurosci. 18*, 347−358.
6. Jansson, L., and Holmdahl, R. (1998) *Inflammation Res. 47*, 290−301.
7. Brzozowski, A. M., Pike, A. C., Daute, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. (1997) *Nature 389*, 753−758.
8. Tanenbaum, D. M., Wang, Y., Williams, S. P., and Sigler, P. B. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 5998−6003.
9. Pike, A. C., Brzozowski, A. M., Hubbard, R. E., Bonn, T., Thorsell, A. G., Engstrom, O., Ljunggren, J., Gustafsson, J. A., and Carlquist, M. (1999) *EMBO J. 18*, 4608−4618.
10. Schwabe, J. W., Chapman, L., Finch, J. T., and Rhodes, D. (1993) *Cell 75*, 567−578.
11. Wikstrom, A., Berglund, H., Hambraeus, C., van den Berg, S., and Hard, T. (1999) *J. Mol. Biol. 289*, 963−979.
12. Fritsch, M., Anderson, I., and Gorski, J. (1993) *Biochemistry 32*, 14000−14008.
13. Emmas, C. E., Fawell, S. E., Hoare, S. A., and Parker, M. G. (1992) *J. Steroid Biochem. Mol. Biol. 41*, 211−219.
14. Attardi, B., and Happe, H. K. (1986) *Endocrinology 119*, 904−915.
15. Kraichely, D. M., Sun, J., Katzenellenbogen, J. A., and Katzenellenbogen, B. S. (2000) *Endocrinology 141*, 3534−3545.
16. Wood, J. R., Greene, G. L., and Nardulli, A. M. (1998) *Mol. Cell. Biol. 18*, 1927−1934.
17. Venyaminov, S. Y., and Yang, J. T. (1996) in *Circular Dichroism and the conformational analysis of biomoleculaes* (Fasman, D., Ed.) pp, 69−107, Plenum Press, New York.
18. Thomas, T., and Kiang, D. T. (1987) *Cancer Res. 47*, 1799−1804.
19. Thomas, T., and Kiang, D. T. (1986) *J. Steroid Biochem. 24*, 505−511.
20. Thomas, T., Gallo, M. A., Klinge, C. M., and Thomas, T. J. (1995) *J. Steroid Biochem. Mol. Biol. 54*, 89−99.
21. Thomas, T., Kulkarni, G. D., Gallo, M. A., Greenfield, N., Lewis, J. S., Shirahata, A., and Thomas, T. J. (1997) *Nucleic Acids Res. 25*, 2396−2402.
22. Lewis, J. S., Thomas, T. J., Shirahata, A., and Thomas, T. (2000) *Biomacromolecules 1*, 339−349.
23. Savitsky, A., and Golay, M. J. E. (1964) *Anal. Chem. 36*, 1627−1639.
24. Bohm, G., Muhr, R., and Jaenicke, R. (1992) *Protein Eng. 5*, 191−195.
25. Brahms, S., and Brahms, J. (1980) *J. Mol. Biol. 138*, 149−178.
26. Smith, L., Greenfield, N. J., and Hitchcock-DeGregori, S. E. (1994) *J. Biol. Chem. 269*, 9857−9863.
27. Moraczewska, J., Greenfield, N. J., Liu, Y., and Hitchcock-DeGregori, S. E. (2000) *Biophys. J. 79*, 3217−3225.

28. Gorski, J., Welshons, W. V., Sakai, D., Hansen, J., Walent, J., Kassis, J., Shull, J., Stack, G., and Campen, C. (1986) *Recent Prog. Horm. Res. 42*, 297−329.
29. Yamamoto, K. R. (1985) *Annu. Rev. Genet. 19*, 209−252.
30. O'Malley, B. W., Tsai, S. Y., Bagchi, M., Weigel, N. L., Schrader, W. T., and Tsai, M. J. (1991) *Recent Prog. Horm. Res. 47*, 1−24.
31. Pratt, W. B., and Toft, D. O. (1997) *Endocr. Rev. 18*, 306−360.
32. Furlow, J. D., Murdoch, F. E., and Gorski, J. (1993) *J. Biol. Chem. 268*, 12519−12525.
33. Boyer, M., Poujol, N., Margeat, E., and Royer, C. A. (2000) *Nucleic Acids Res. 28*, 2494−2502.
34. Katzenellenbogen, J. A., O'Malley, B. W., and Katzenellenbogen, B. S. (1996) *Mol. Endocrinol. 10*, 119−131.
35. McKenna, N. J., Lanz, R. B., and O'Malley, B. W. (1999) *Endocr. Rev. 20*, 321−344.
36. Xu, J., Liao, L., Ning, G., Yoshida-Komiya, H., Deng, C., and O'Malley, B. W. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 6379−6384.
37. Llopis, J., Westin, S., Ricote, M., Wang, Z., Cho, C. Y., Kurokawa, R., Mullen, T. M., Rose, D. W., Rosenfeld, M. G., Tsien, R. Y., Glass, C. K., and Wang, J. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 4363−4668.
38. Chan, C. M., Lykkesfeldt, A. E., Parker, M. G., and Dowsett, M. (1999) *Clin. Cancer Res. 5*, 3460−3467.
39. Nardulli, A. M., and Shapiro, D. J. (1992) *Mol. Cell. Biol. 12*, 2037−2042.
40. Nardulli, A. M., Greene, G. L., and Shapiro, D. J. (1993) *Mol. Endocrinol. 7*, 331−340.
41. Spolar, R. S., and Record, M. T., Jr. (1994) *Science 263*, 777−784.
42. Johnson, N. P., Lindstrom, J., Baase, W. A., and von Hippel, P. H. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 4840−4844.
43. Werner, M. H., Gronenborn, A. M., and Clore, G. M. (1996) *Science 271*, 778−784.
44. Cooper, T. M., and Woody, R. W. (1990) *Biopolymers 30*, 657−676.
45. Metivier, R., Petit, F. G., Valotaire, Y., and Pakdel, F. (2000) *Mol. Endocrinol. 14*, 1849−1871.
46. Perissi, V., Staszewski, L. M., McInerney, E. M., Kurokawa, R., Krones, A., Rose, D. W., Lambert, M. H., Milburn, M. V., Glass, C. K., and Rosenfeld, M. G. (1999) *Genes Dev. 13*, 3198−3208.
47. Barkhem, T., Carlsson, B., Nilsson, Y., Enmark, E., Gustafsson, J., and Nilsson, S. (1998) *Mol. Pharmacol. 54*, 105−112.
48. Mader, S., Chambon, P., and White, J. H. (1993) *Nucleic Acids Res. 21*, 1125−1132.
49. Henderson, B. E., Ross, R. K., and Pike, M. C. (1993) *Science 259*, 633−638.
50. Clarke, R., Dickson, R. B., and Lippman, M. E. (1992) *Crit. Rev. Oncol. Hematol. 12*, 1−23.
51. Jordan, V. C. (1995) *Breast Cancer Res. Treat. 36*, 267−285.
52. Katzenellenbogen, B. S., Montano, M. M., Ekena, K., Herman, M. E., and McInerney, E. M. (1997) *Breast Cancer Res. Treat. 44*, 23−38.
53. Katzenellenbogen, B. S., Montano, M. M., Ediger, T. R., Sun, J., Ekena, K., Lazennec, G., Martini, P. G., McInerney, E. M., Delage-Mourroux, R., Weis, K., and Katzenellenbogen, J. A. (2000) *Recent Prog. Horm. Res. 55*, 163−193.

BI002846L