Conformational dynamics of estrogen receptors α and β as revealed by intrinsic tryptophan fluorescence and circular dichroism

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Abstract

Estrogen receptors (ERα and ERβ) are ligand-activated nuclear receptors that mediate the action of estrogens. These receptors activate transcription by similar mechanism(s), although the overall amino acid sequence identity is only 47%. In order to compare the structural and conformational features of ERα and ERβ, we monitored their intrinsic tryptophan fluorescence during thermal unfolding. The 50% unfolding temperatures (Tm) of ERα and ERβ were 39±1 and 40±2°C, respectively. Estradiol had no significant effect on the Tm of ERα or ERβ. In contrast, binding of the estrogen-response element increased the Tm of ERα and ERβ by 10 °C. Thermal unfolding of estradiol-bound ERα and ligand-free ERβ showed two-step transitions, with the formation of intermediates that were stable between 36–48 and 34–42°C, respectively. We confirmed the presence of intermediate states during thermal unfolding by circular dichroism spectroscopy. Atomic force microscopy showed that the ERβ intermediate consisted of discrete globular particles, whereas the ERα intermediate showed a speckled appearance, with sparse well-defined particles. Fluorescence-quenching studies showed the presence of two classes of tryptophan in unliganded ERα and ERβ. Binding of estradiol to ERβ exposed its tryptophans, whereas estradiol reduced the accessibility of the tryptophans of ERα. Our results illustrate the differential effects of ligands on the unfolding of ERα and ERβ, and identify partially unfolded intermediates. Differences in the conformational flexibility and stability of ERα and ERβ may represent functional differences of ligand-bound ERs in recruiting coactivator proteins and initiating transcription.

Journal of Molecular Endocrinology (2005) 35, 211–223

Introduction

The estrogen receptors (ERα and ERβ) are ligand-dependent transcriptional activators belonging to the superfamily of nuclear receptors (Gustafsson et al. 1999, McKenna et al. 2002, Katzenellenbogen & Frasor 2004, Thomas et al. 2004, McDonnell 2005). ERs mediate the function of the steroid hormone, estradiol (E2), in both males and females. ERs and other nuclear receptors share a common modular structure consisting of five domains, named A/B, C, D, E and F, and some key functions have been assigned to each domain (Metzger et al. 1995, Tsai et al. 2004). The N-terminal A/B domain contains the ligand-independent transcription activation function 1 (AF-1; Metzger et al. 1995, Tsai et al. 2004). The C domain has a characteristic zinc-finger structure responsible for the binding of estrogen-response elements (EREs; Danielian et al. 1992, Krieg et al. 2004). The D domain appears to be a hinge region that can modulate the DNA-binding ability of the receptor (Kumar et al. 1986). The E and F domains are involved in the ligand-binding function and exhibit strong ligand-dependent activation function (AF-2; Kumar et al. 1986, Brooks & Skafer 2004). ERα and ERβ share a modest overall sequence identity (47%), although in the DNA-binding domain (DBD) and ligand-binding domain (LBD) the amino acid identity is 97 and 59%, respectively (Kuiper et al. 1996, Mosselman et al. 1996, Tremblay et al. 1997). In addition, the A/B domain is shorter in ERβ than in ERα (Kuiper et al. 1996). Despite these differences, ERα and ERβ have similar functions as E2-induced transcriptional activators, although cellular factors and promoter context determine the degree of transactivation (Couse et al. 1997, Strom et al. 2004, Ramsey et al. 2004).

E2 binds to ERα and ERβ with similar binding affinities, leading to conformational changes that result in the dissociation of heat-shock proteins and dimerization (Kuiper et al. 1996). ERα and ERβ may form homo- or heterodimers and bind to the same ERE sequence present in estrogen-responsive genes. However, ERα homodimers are more potent transcriptional activators than the heterodimers or ERβ homodimers (Matthews & Gustafsson 2003). Thermodynamic and
structural studies show that specific contacts between transcriptional activator proteins and DNA are associated with conformational changes in protein, DNA, or both (Pabo and Sauer 1992, Spolar and Record 1994, Greenfield et al. 2001, Margeat et al. 2003). Although the crystal-structure studies of LBD and DBDs of ERα and ERβ are available (Brzozowski et al. 1997, Pike et al. 1999, Manas et al. 2004) and genetic studies have defined amino acid requirements and function (Wood et al. 2001, Metivier et al. 2002, Nettles et al. 2004), physicochemical studies on the conformation of full-length ERα and ERβ are limited (Greenfield et al. 2001, Margeat et al. 2003, Bouter et al. 2005).

To gain insight into the role of E2 and ERE in regulating gene expression, we examined the structural and conformational changes involved in E2-ER and ER-ERE recognition. Since ERα and ERβ are targets for drug development for breast cancer and other diseases (Thomas et al. 2004, Turgeon et al. 2004, McDonnell 2005), detailed information on the structural differences and similarities of these two proteins might help to determine the mechanisms by which these receptors exert their biological action and facilitate drug discovery. In addition, the folding/unfolding profile of ERα and ERβ is of interest as protein misfolding and functional abnormalities are linked to human diseases (Yon 2002, Castro-Fernandez et al. 2005, Kamagata et al. 2004). Folding/unfolding studies also give insight into the relationship between amino acid sequence, three-dimensional structure, and function. We examined the unfolding of ERα and ERβ using temperature as the perturbant. We used the intrinsic tryptophan fluorescence and circular dichroism (CD) spectroscopy to detect the thermal stability and unfolding profile of the proteins. We found that the ERE significantly stabilized both ERα and ERβ. The ligand-free ERβ and the ERα-E2 complex exhibited the most stable partially unfolded state during thermal unfolding. Quenching studies using acrylamide revealed dissimilar properties of ERα and ERβ. Atomic force microscopy (AFM) of the partially unfolded state of ERβ showed well-defined spherical particles.

Materials and methods

Materials

Full-length recombinant ERα and ERβ were purchased from Invitrogen (Carlsbad, CA, USA). The specific ligand-binding activities of ERα and ERβ were 2800 and 3500 pmol/ml, respectively, as determined by hydroxylapatite assay (Maaroufi & Leclercq 1994). The purity of ERα and ERβ was tested by SDS/PAGE, followed by Coomassie Brilliant Blue staining. ERα and ERβ exhibited bands at 66 and 53 kDa, respectively. E2 was purchased from Sigma Chemical Co. (St Louis, MO, USA).

HPLC-/PAGE-purified oligodeoxyribonucleotides (ODNs) were purchased from Oligos, Etc. (Wilsonville, OR, USA). 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 use in our experiments. An ODN having a similar percentage of GC content, arranged in a scrambled sequence, was used as a control ODN (Thomas et al. 1997). A mutant ERE with a 2 bp difference and an ODN with an ERE half-site were also used. Base sequence (upper strand) of the ERE, mutant, and control ODNs is listed below, with the palindromic ERE and mutant sequences underlined: ERE, 5′-GATCCAGGTAGTGACCTGAGCTAAATAACACATTCAG-3′; mutant ERE, 5′-GATCCAGGTAGTGACCTGAGCTAAATAACACATTCAG-3′; control ODN, 5′-AAAGCTCGCTTGAGCATGGTGAAACGTAAATAACACATTCAG-3′; ERE half-site ODN, 5′-GATCCAGGTAGTGACCTGAGCTAAATAACACATTCAG-3′.

Fluorescence measurements

The stock solution of ERα/ERβ was diluted to 50 nM, with a final buffer concentration of 50 mM Tris/HCl (pH 8), 150 mM KCl, 2 mM dithiothreitol (DTT), 1 mM EDTA, and 10% glycerol. The ERE solution used in our experiments was freshly made before each experiment. Complementary single strands were prepared in annealing buffer (10 mM Tris/HCl and 50 mM NaCl, pH 7.5), and equimolar concentrations mixed in the same buffer and heated in a water bath for ∼10 min. The solution was allowed to cool to 22°C and incubated at this temperature for 2 h. E2 and ERE concentrations used in our experiments were 1 µM. Before measuring the fluorescence intensity, the E2/ERE/ER reaction mixture (total volume 300 µl) was incubated for 1 h on ice.

Fluorescence experiments were carried out using a FluoroMax-2 spectrofluorometer. All measurements were done in a 10 mm quartz cuvette. The excitation and emission slits were set at 5 nm. The intrinsic tryptophan fluorescence was measured using an excitation wavelength of 295 nm to avoid tyrosine emission. Emission spectra were recorded from 310–380 nm, using 5 nm band pass for both excitation and emission. The sample temperature was controlled by a Neslab circulating-water bath. For temperature studies of ER, samples were held at each temperature for 6 min, and then the fluorescence emission was recorded. The thermal unfolding of ERα and ERβ was studied by recording the tryptophan fluorescence emission at 310–380 nm as a function of temperature. All the spectra were corrected for baseline and for the presence of E2 or ERE; however, E2 and ERE did not show any fluorescence at the range of study. The fraction of unfolded ERα/ERβ at each temperature was calculated using the following equation (Pace 1986):

\[ F(t)/F(0) = \exp(-k(t-t_0)) \]

where F(t) is the fluorescence intensity at time t, F(0) is the fluorescence intensity at time t_0, and k is the rate constant of unfolding.


\[ f_u = (F_{\text{obs}} - F_u)(F_d - F_u) \]  

where \( f_u \) is the amount of unfolded fraction at a particular temperature, \( F_{\text{obs}} \) is the fluorescence intensity at the temperature, \( F_u \) is the fluorescence intensity of native protein, and \( F_d \) is the fluorescence intensity of the denatured (totally unfolded) protein. \( T_M \) is the temperature at which 50% protein is unfolded, and calculated from a plot of \( f_u \) against temperature.

**CD spectroscopy**

The stock solution of ER\( \alpha \) and ER\( \beta \) was diluted 1:3 for CD measurements. Final buffer concentration for ER\( \alpha \) and ER\( \beta \) was 50 mM Tris/HCl (pH 8), 150 mM KCl, 2 mM DTT, 0.3 mM EDTA, 0.3 mM sodium orthovanadate, and 10% glycerol. Data were collected on an Aviv model 215 CD spectrometer, fitted with a five-compartment thermal equilibration chamber. Spectra were collected from 260 to 200 nm at 0.5 nm intervals, collecting data for 2 s at each point for temperatures from 20 to 70 °C with 5 °C intervals, except for the intermediate temperature range (30–40 °C) where the spectra were collected at 2 °C intervals. Data were smoothed using the method of Savitsky and Golay (1964) and corrected for the contribution of the cells. Spectra were also corrected for the contribution of the ERE in samples containing ER and the ERE. The CD data were analyzed using the convex constraint algorithm (CCA) analysis program (Perczel et al. 1991) to determine the minimum number of basis spectra needed to reconstruct the data set obtained as a function of temperature.

**AFM**

The AFM images were obtained using a Nanoscope IIIA equipment (Digital Instruments, Santa Barbara, CA, USA) in tapping mode, operating in ambient air. A 125 µm long rectangular silicon-tip assembly was used with a spring constant of 40 Nm. The images were generated by the change in amplitude of the oscillation of the tip, as it interacted with the sample. The height differences on the surface of the sample are indicated by the color code, as shown in the AFM images. ER\( \alpha \)/ERE protein concentration of 50 µg/ml was used for the AFM measurements. AFM measurements were done for three different temperatures (22, 37, and 65 °C). Before depositing on the mica surface, samples were kept at the required temperatures for 15 min. Aliquots (3 µl) of these samples were deposited on a freshly cleaved mica surface. Before AFM measurements, the mica surface was dried at room temperature for 5 min, rinsed with two or three drops of nanopure water (Barnstead), and dried under a flow of nitrogen.

**Fluorescence quenching with acrylamide**

Quenching studies were conducted using acrylamide or KI as the quencher for both ER\( \alpha \) and ER\( \beta \) in the presence or absence of ligands (E2, ERE, or E2+ERE). The stock ER\( \alpha \) or ER\( \beta \) solution was diluted to 50 nM using Tris/HCl buffer (pH 7.5) containing 150 mM KCl, 2 mM DTT, 1 mM EDTA, and 10% glycerol. For acrylamide quenching, aliquots of a freshly prepared 5 M acrylamide stock solution were added to achieve the required concentration. The quenching was monitored as a decrease in the intensity of intrinsic tryptophan fluorescence emission with increasing concentration of quenchers. Prior to recording spectra, the sample was mixed gently and incubated for 5 min at 22 °C. The fluorescence spectra presented herein are averages from three scans. The spectra were corrected for background and dilution. For KI quenching experiments, aliquots of a freshly prepared KI stock solution were added to samples containing 50 nM ER\( \alpha \) or ER\( \beta \) to achieve the indicated KI concentration. KCl, which does not quench fluorescence, was added to maintain a constant salt concentration. Fluorescence-quenching data were fitted to the classical Stern–Volmer equation. Effective Stern–Volmer constants (\( k_{SV} \)) were obtained from the fluorescence data according to the Stern–Volmer equation for dynamic quenching (Eftink & Ghiron 1976):

\[ \frac{F_0}{F} = 1 + k_{SV}[Q] \]  

where \( F_0 \) and \( F \) are the fluorescence intensities in the absence and presence, respectively, of a given concentration of quencher \( [Q] \). \( k_{SV} \) is the quenching constant and is obtained from the slope of the linear Stern–Volmer plot. The value for \( k_{SV} \) can be considered as a reliable reflection of the bimolecular collisional constant for collisional quenching of the tryptophan since \( k_{SV} = k_q \tau_0 \), where \( k_q \) is the bimolecular collisional constant and \( \tau_0 \) is the lifetime constant in the absence of the quencher. However, if all tryptophan residues are not equally accessible to the quencher, a modification of the Stern–Volmer plot can be described by the Lehrer equation (Lehrer 1971):

\[ F_0(F_0 - F) = 1/k_{SV}[Q] + 1/f_a \]  

where \( (F_0 - F) \) refers to the change in fluorescence intensity on addition of the quencher and \( f_a \) refers to the fraction of tryptophans accessible to the quencher.

**Results**

**Thermal unfolding of ER\( \alpha \) and ER\( \beta \) monitored by tryptophan fluorescence**

Figure 1 shows the tryptophan emission fluorescence spectra of ER\( \alpha \) and ER\( \beta \) with increasing temperature,
Figure 1 Fluorescence emission spectra of ERα and ERβ (50 nM) with increasing temperature. The temperature increased from 20 to 90°C: 1 on the figure indicates 20°C, 20 indicates 90°C. (A) Unliganded ERα; (B) ERα in the presence of E2; (C) ERα in the presence of ERE; (D) unliganded ERβ; (E) ERβ in the presence of E2; (F) ERβ in the presence of ERE. Spectra are representative of three separate experiments. a.u., arbitrary units.
from 20 to 90 °C. ERα and ERβ showed similar emission spectra (Fig. 1A to 1F). The highest intensity was observed at 20 °C. The intensity at 337–340 nm (λmax) decreased as the temperature increased, indicating the exposure of tryptophans and quenching of fluorescence emission by the solvent. Significant red shifts (Δλ ≃ 5 nm) were observed at higher temperatures, during the unfolding of ERβ under all conditions and ERα in the presence of E2. ERα unfolded via an intermediate state in the presence of E2 (Fig. 1B); the temperature-dependent decrease in intensity of emission halted at this stage and then gradually decreased at higher temperatures. Unfolding in the absence of E2 (Fig. 1A) showed a less distinct intermediate. However, an intermediate state of ERα was not detectable in the presence of the ERE (Fig. 1C) or E2+ERE (not shown).

Figure 1D shows the tryptophan emission fluorescence spectra of ERβ in the absence of any ligand. ERβ unfolded via a distinct intermediate state. However, unfolding of ERβ did not show an intermediate in the presence of E2 (Fig. 1E). There were also no distinct intermediates for ERβ in the presence of the ERE (Fig. 1F) or E2+ERE (not shown). These results indicate that specific binding of E2 and ERE converts ERβ to a conformational state where an increase in temperature leads to a single-step unfolding and loss of tryptophan fluorescence.

The unfolded fractions of ERα and ERβ were calculated using eqn 1, and plotted against temperature. A two-step unfolding was observed for ERα in the presence of E2 (36–48 °C; Fig. 2B) and ERβ in the absence of E2 (34–42 °C; Fig. 2E), indicating that intermediate conformational states are stable over a range including physiological temperature. Although the thermal unfolding profile of ERα was similar to that of ERβ (Fig. 2B), showing a two-step process, that of ERβ was consistent with a single-step unfolding. In the presence of the ERE, ERβ unfolding showed a single-phase transition (Fig. 2 G), from the native to unfolded state. We also studied the unfolding of ERα in the presence of a mutant ERE (Fig. 2D and H) or a control ODN (results not shown). ERβ exhibited unfolding profiles similar to their unliganded state, indicating that mutant ERE/control ODN did not affect the thermal unfolding process. In the case of ERα, the intermediate was not detectable in the presence of mutant/control ODN and barely detectable in the presence of the ERE (Fig. 2C and D). These results show that ERα conformational intermediate was stabilized by E2 but not by the ERE or mutant ERE, whereas the intermediate state of ERβ was lost in the presence of E2 or ERE. Thus, ERα and ERβ show distinct differences in the conformational flexibility and stability of the native and ligand-bound states.

Table 1 presents the midpoint (50%) of unfolding temperature (Tm) values of ERα and ERβ determined from the change in fluorescence as a function of temperature. Although the unfolding profiles showed differences in the stability of intermediates, overall stabilities of ERα and ERβ (as represented by Tm values) were generally similar. In the absence of ligands, the Tm values of ERα and ERβ were 39 ± 1 and 40 ± 2 °C, respectively. There was no significant change in the Tm values of ERα and ERβ in the presence of E2. In contrast, the presence of the ERE increased the Tm by 10°C. These results suggested that the ERE binding provoked a thermally stable conformation in both ERα and ERβ. We also observed a similar stabilization of ERα and ERβ in the presence of E2+ERE. However, the control ODN and mutant ERE did not increase the Tm of ERα or ERβ, demonstrating the specificity of binding of ERα and ERβ to the ERE.

The palindromic ERE is known to bind to dimeric form of ERα and ERβ. However, native ERαs include
functional half-site EREs, direct repeats, and other variations (Klinge 2001). To see whether a half-site ERE is sufficient to induce conformational changes in ER$\alpha$/ER$\beta$, we examined the effect of an ODN with the ERE half-site on the stability of ER$\alpha$ and ER$\beta$ by tryptophan fluorescence measurements. Figure 3 shows the results of these studies. We found that ER$\alpha$ was stabilized by ODN containing the ERE half-site, yielding a TM of 49.7 ± 1.5 °C. This result indicates that a dimeric binding site is not required for the stabilization of ER$\alpha$ by ERE. In contrast, ER$\beta$ was not stabilized by the presence of ODN containing half-site ERE (TM = 39 ± 1.5 °C), indicating that dimeric binding site is important for the stabilization imparted by ERE.

**Table 1 Thermal unfolding temperatures (TM) of ER$\alpha$ and ER$\beta$ in the presence/absence of ligands**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>ER$\alpha$ TM (°C)*</th>
<th>ER$\beta$ TM (°C)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>39±1.0</td>
<td>40±2</td>
</tr>
<tr>
<td>E2</td>
<td>38±2.7</td>
<td>40±2</td>
</tr>
<tr>
<td>ERE</td>
<td>50±1.3</td>
<td>50±1.5</td>
</tr>
<tr>
<td>ERE+E2</td>
<td>47±4.1</td>
<td>51±1.0</td>
</tr>
<tr>
<td>ERE Half-site+E2</td>
<td>49±1.5</td>
<td>39±1.5</td>
</tr>
<tr>
<td>ERE+mERE</td>
<td>42±4.7</td>
<td>39±5.0</td>
</tr>
<tr>
<td>Control ODN</td>
<td>39±1.0</td>
<td>40±1.0</td>
</tr>
</tbody>
</table>

*The overall TM values (temperature at which 50% unfolding of the proteins occurred) were obtained from the plot of unfolded fraction versus temperature. Mean±S.D. from three experiments are presented. mERE, mutant ERE.

**Figure 3 Thermal unfolding profile of ER$\alpha$ and ER$\beta$ in the presence of E2 and the ERE half-site ODN.** Tryptophan fluorescence spectra were recorded at different temperatures from 20 to 90 °C. Unfolded fractions of ER$\alpha$ and ER$\beta$ were calculated and plotted against temperature. Data are means±S.D. from three separate experiments.

We further examined the thermal unfolding of ER$\alpha$ and ER$\beta$ by CD spectroscopy. Figure 4 shows the temperature-dependent CD spectra for ER$\alpha$ and ER$\beta$ from 200 to 260 nm, between 20 and 70 °C. The CD spectra of ligand-free ER$\alpha$ and E2-bound ER$\alpha$ are shown in Fig. 4A and B, respectively. The CD spectrum of ER$\alpha$ showed negative-ellipticity bands at 225 and 208 nm, characteristic of a protein with a high $\alpha$-helical content and some turns. The bands decreased sharply as the temperature increased from 20 to 30 °C and then gradually from 30 to 70 °C. Analysis of the unfolding data using the CCA algorithm showed that at least three component curves were needed to characterize the unfolding (Fig. 5A–D). As the temperature increased, the initial component curves rapidly disappeared and were replaced by a curve that lost about 50% of the total ellipticity but retained a large helical component, as manifested by its negative ellipticity at 222 and 208 nm. In the absence of E2, the intermediate component of ER$\alpha$ showed peak levels at 30 °C (Figs. 5A and B). In the presence of E2, the intermediate component showed a broad range of stability between 30 and 45 °C (Fig 5C and D), suggesting a more stable intermediate. At temperatures higher than 50 °C the unfolded protein showed negative single-band ellipticity near 220 nm, indicating a high content of $\beta$-pleated sheet structure, characteristic of aggregation as the protein unfolded further.

**Figure 4C and D show the spectra of ER$\beta$ and E2-bound ER$\beta$.** The spectra of ER$\beta$ showed only minor changes between 20 and 30 °C, indicating relatively higher stability of ER$\beta$ at this temperature compared with ER$\alpha$. As in the case of ER$\alpha$, CCA deconvolution analysis of the temperature-dependent changes in the spectra (Fig. 5E–H) showed that at least three curves were needed to reconstruct the data set. The native state was lost above 34 °C, and was replaced by a partially folded structure that retained some helical content. This state is present at temperatures where the intermediate state was observed by tryptophan fluorescence. The intermediate state appeared to be stable between 34 and 42 °C (Fig. 5F). At higher temperatures, the protein began to aggregate. The CD spectrum of the third component is characteristic of an unfolded protein that aggregates to give some $\beta$-pleated sheet. In the presence of E2, the intermediate component began to disintegrate at 36 °C (Fig. 5 G and H), indicating a lower stability of this component, compared with the structure in the absence of E2.

**AFM**

We next conducted AFM experiments to determine the morphology of the intermediate states of ER$\alpha$/ER$\beta$. The AFM experiments were done at room temperature
(22 °C) and a temperature at which the intermediates were observed (37 °C). Figure 6 shows representative AFM images obtained for ERα and ERβ. Table 2 presents the dimensions of the particles, measured using Nanoscope IIIA software. Figures 6A and B show AFM images of ERα-E₂ complexes incubated at 22 and 37 °C, respectively. At 22 °C, the ERα-E₂ sample showed an amorphous protein structure, with <10% of particles of 74 ± 34 nm diameter and 2·8 ± 1·7 nm height. After incubation at 37 °C, ERα-E₂ samples showed molecules with a speckled appearance and particles with 37 ± 21 nm diameter and 1·5 ± 1 nm height. Figure 6C shows AFM image of a sample of ERα without E₂. In this case, images showed mostly amorphous aggregates. These results indicate that ERα-E₂ complexes formed compact particles, although these particles consisted of only about 20% of the aggregate images observed.

AFM studies of ERβ at 22 °C (Fig. 6D) showed that the majority of molecules were in an amorphous state, with ~25% particles of 69 ± 28 nm diameter and 2·0 ± 1·4 nm height. Samples of ERβ incubated at 37 °C (Fig. 6E), however, showed compact globular nanoparticles and 90% of them had a diameter of 30 ± 7 nm and 1·1 ± 0·5 nm height. In the presence of E₂, however, the majority of ERβ molecules were present as aggregates (Fig. 6F). These results indicate that compact structures exist during the unfolding of ERα and ERβ. In the absence of E₂, ERβ intermediate structures appeared to be more stable than that of ERα at 37 °C.

Quenching of tryptophan fluorescence of ERα and ERβ with acrylamide

Acrylamide is a neutral water-soluble quencher that can permeate into hydrophobic environments of proteins. As a nonionic quencher, it gives information about the exposed hydrophobic surfaces, as it penetrates the hydrophobic interiors. The fluorescence spectra of ERα/ERβ were recorded with increasing concentrations of acrylamide. The resulting $F_0/F$ values were plotted against the concentration of acrylamide. The
Stern–Volmer plots of ERα and ERβ quenching, obtained in the presence and absence of ligands, are given in Fig. 7. Table 3 shows the Stern–Volmer constants ($K_{SV}$) and $f_a$ values of ERα and ERβ in the presence/absence of ligands. The $K_{SV}$ values were obtained by fitting the linear part of the curve. The slope of the curve gives the $K_{SV}$ values according to eqn 2. The $f_a$ values (amount of exposed tryptophans) are obtained from the modified Stern–Volmer plot (not shown) according to eqn 3. Table 3 shows $K_{SV}$ and $f_a$ values under different conditions. Unliganded ERα and ERβ have $K_{SV}$ values of $4.22 \pm 0.5$ and $5.6 \pm 0.15 \text{ M}^{-1}$, respectively, with a slightly higher $K_{SV}$ value for ERβ. Fully exposed tryptophan residues have $K_{SV}$ values of $8.9 \text{ M}^{-1}$, whereas $K_{SV}$ values for buried or inaccessible tryptophan residues are lower and can be close to zero (Eftink & Ghiron 1981). The Stern–Volmer plots of unliganded ERα and ERβ are similar, non-linear with a downward curvature at higher concentrations of acrylamide, suggesting that two classes of tryptophans exist in ERα and ERβ, one of which is less accessible to the quencher.

ERβ had similar quenching patterns in the ligand-free state and for ERα-E2-ERE complex, with $K_{SV}$ values of $4.22 \pm 0.5$ and $4.52 \pm 0.9 \text{ M}^{-1}$, respectively. However, the $K_{SV}$ value was considerably lower in the presence of E2 ($2.5 \pm 0.3 \text{ M}^{-1}$). The $K_{SV}$ value of the ERα-ERE complex was higher than that of native ERα. These results indicate that E2 binding reduced the accessibility of tryptophans in ERα, whereas ERE binding increased their accessibility. The $K_{SV}$ value of the ERα-ERE complex was in between that of the ERα-E2 and ERβ-ERE complexes. The $f_a$ values for ERα under all the conditions were $\sim 86\%$ or less, except for ERα-ERE, for which the value was 1 (100%).

In the presence of E2, $K_{SV}$ values were greater than 9 M$^{-1}$ for ERβ, regardless of the presence of the ERE, and showed an increase compared with the $K_{SV}$ value in the absence of the ligand. In these cases (ERβ-E2 and ERβ-E2-ERE) Stern–Volmer plots were similar with an upward curvature; that is, towards the y axis, suggesting that the tryptophans were easily accessible to the quencher. The inclination toward the y axis indicates the presence of two types of quenching: static and dynamic (Eftink & Ghiron 1976). Therefore, we plotted apparent quenching constant ($K_{app}$) against concentration of quencher (not shown) to separate the static and dynamic components. Both ERβ-E2-ERE and ERβ-E2 had positive slopes, indicating the close proximity of the quencher and the tryptophans. The Stern–Volmer plot of ERβ-ERE showed a similar quenching pattern to that of native ERβ. $K_{SV}$ values were similar in the presence and absence of the ERE. Results of KI quenching (data not presented) of ERα and ERβ in the presence of E2 and ERE were similar to those of acrylamide quenching, indicating similar patterns for ionic and nonionic quenchers.

**Discussion**

Our study of the thermal unfolding of ERα and ERβ in the presence and absence of E2 and ERE shows common features and differences in the conformational...
states of ERα and ERβ. Unliganded ERα and ERβ were dissimilar in their thermal unfolding pattern, showing a relatively stable intermediate structure for ERβ. However, the overall stability of ERα and ERβ in the presence of E2 or ERE was similar. Complex formation with the ERE increased the $T_M$ of both ERα and ERβ from $\sim 40$ °C to $\sim 50$ °C. A control ODN or mutant ERE with a 2 bp difference was unable to stabilize ERα or ERβ, indicating the nucleotide specificity of the interaction. Tryptophan fluorescence studies showed that native ERβ unfolded with a stable intermediate state at physiological temperature, in the absence of ligands or in the presence of nonspecific DNA sequence. However, native ERα was extremely labile and binding of E2 provided some stability to the partially unfolded structure. Binding of the ERE provoked ERα and ERβ into a conformational state that could unfold by a single thermal transition. Quenching studies showed that the tryptophan environment of ERβ underwent changes due to E2 or E2+ERE binding, leaving highly exposed tryptophans in the ligand-bound ERβ. In contrast, binding of E2 to ERα induced a conformational state with less accessible tryptophans. AFM studies showed

![Figure 6](image-url) AFM images of native and intermediate states of ERα and ERβ. (A) ERα-E2 complex at 22 °C; (B) ERα-E2 complex incubated at 37 °C for 15 min; (C) ERα in the absence of E2 incubated at 37 °C; (D) ERβ at 22 °C; (E) ERβ incubated at 37 °C; (F) ERβ-E2 complex incubated at 37 °C. Scale bars, 500 nm. Similar data were obtained in two separate experiments.

### Table 2

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<th>Sample</th>
<th>Native particle dimensions (nm)</th>
<th>Intermediate particle dimensions (nm)</th>
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<td></td>
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<tr>
<td>ERα-E2</td>
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Native particles represent samples at 22°C. Intermediate particles represent samples heated at 37°C for 15 min before depositing on the mica surface. Diameter and height of ER particles were determined using Nanoscope IIIA software. For ERβ intermediate particles, dimensions are from 432 particles. Other dimensions are from ~20 particles. Images from other conditions were either not detected or of poor quality, probably due to changes in charge conditions of the protein and/or poor attachment to mica.

![Figure 7](image-url) Stern–Volmer plots obtained for ERα (○) and ERβ (●) for quenching with acrylamide. (A) Unliganded ERα and ERβ; (B) ERα and ERβ in the presence of E2; (C) ERα and ERβ in the presence of ERE; (D) ERα and ERβ in the presence of E2+ERE. Data are mean±s.d. from three separate experiments.
that incubation at physiological temperature allowed ERβ to form compact, globular structures in abundance, whereas ERα structures disintegrated rapidly. These results indicate distinct differences in the structure and conformation of ERα and ERβ in their ligand-free states, in the partially unfolded intermediates, as well as in E2-induced conformational transitions.

ERα and ERβ proteins have five and seven tryptophans, respectively, of which three tryptophans are located in the LBD at equivalent positions of ERα and ERβ, as aligned by amino acid homology (Kuiper et al. 1996, Mosselman et al. 1996). Another tryptophan is located in the LBD of ERβ at position 386, and the other three are located in the A/B region (positions 27, 54, and 111). In ERα, one tryptophan is located in the DBD (position 200) and one in the hinge region (position 291) between DBD and LBD. Temperature-dependent changes in tryptophan fluorescence appear to indicate the mean of the fluorescence quenching initiated by solvation. The presence of E2 did not stabilize ERα against temperature-induced fluorescence quenching, but the presence of the ERE stabilized ERα and ERβ. Since these effects are similar in ERα and ERβ, regardless of the different positions of the tryptophans, temperature-dependent effects can be considered to be due to overall changes in the conformation, rather than changes in individual tryptophans. However, presence and stability of intermediate structures of ERα/ERβ were different in the presence and absence of E2, and these differences were largely confirmed by independent techniques.

Tryptophan fluorescence studies using an ERE half-site ODN also indicate differences between ERα and ERβ. ERα was stabilized by both the palindromic ERE and the half-site ERE, while ERβ was stabilized by the palindromic ERE, but not the half-site ERE (Table 1). Previous studies using electrophoretic mobility-shift assays showed that both ERα and ERβ are able to bind to half-site ERE as dimers (Lopez et al. 2002, van de Stolpe et al. 2004). Thus differences in stability are unlikely to be due to increased stabilization of homodimers in the presence of the palindromic ERE compared with half-site ERE. Therefore, our results suggest higher conformational flexibility of ERα in the presence of ERE or ERE-like sequences, compared with ERβ.

Acrylamide-quenching studies show that unliganded ERα and ERβ have similar quenching behavior with $K_{SV}$ values of 4·2 and 5·6 M$^{-1}$, respectively. The downward curvature of the Stern–Volmer plot of acrylamide quenching indicates two classes of tryptophans, with different accessibility to the quencher. The $f_a$ values of ERα/ERE indicate that about 14–15% of total tryptophans are inaccessible to the quencher. Quenching results show that the tryptophan environment of ERα and ERβ undergoes contrasting changes due to the binding of E2. E2 binding reduces exposed tryptophans compared with the ligand-free ERα, whereas it increases the accessibility of tryptophans in ERβ ($f_a = 1·0$). These results suggest that a relatively closed conformation of the ligand-binding pocket of ERα LBD is stabilized in the presence of E2, but that such a stabilization does not occur for ERβ. This idea is also supported by the lack of stable intermediate structures of ERβ in the presence of E2.

$K_{SV}$ values were higher (>9 M$^{-1}$) for E2$^-$ or E2+ERE-bound ERβ and the Stern–Volmer plot has an upward curvature, indicating that the tryptophans in this case are fully accessible to the quencher. The $f_a$ value (1·0) also indicates that all the tryptophans are fully exposed in these cases. However, the quenching data show that tryptophans are not fully accessible in ERβ-ERE complex in the absence of E2. Although binding of the ERE stabilizes ERβ in terms of T$m$-tryptophans remain buried in its presence, indicating that ERE binding alone is not sufficient to change the tryptophan environment. The absence of tryptophans in the DBD of ERβ may also explain the lack of changes in the tryptophan environment due to ERE in the absence of E2. In contrast, binding of ERE to ERα exposes tryptophans fully. Previous studies using protease sensitivity have also demonstrated independent conformational changes induced by E2 or the ERE in ERα and ERβ (Loven et al. 2001, Yi et al. 2002b).

CD studies are consistent with AFM and tryptophan fluorescence studies on the relatively high stability of intermediates formed during the unfolding of the ERα-E2 complex and the ligand-free ERβ. CD studies also showed the presence of intermediates during unfolding of both ERs under other conditions as well, although the intermediate states were less stable and not well defined. Proteins with >100 amino acid residues tend to unfold through an intermediate form, known as a molten globule or compact globule, with fluctuating tertiary structure (Ptitsyn 1998, Kamagata et al. 2004). Although the shape of the unfolding curve with an

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**Table 3** $K_{SV}$ values for acrylamide quenching of ERα and ERβ

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$K_{SV}$ value</th>
<th>$f_a$*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ERα</td>
<td>ERβ</td>
</tr>
<tr>
<td>Control</td>
<td>4·2±0·5</td>
<td>5·59±0·15</td>
</tr>
<tr>
<td>E2</td>
<td>2·5±0·3</td>
<td>9·5±0·8</td>
</tr>
<tr>
<td>ERE</td>
<td>6·4±1·5</td>
<td>4·84±0·3</td>
</tr>
<tr>
<td>ER+E2</td>
<td>4·52±0·9</td>
<td>10·7±0·2</td>
</tr>
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</table>

*Fraction of exposed tryptophan ($f_a$) values were obtained from the modified Stern–Volmer equation. Means±S.D. from three experiments are presented. The $K_{SV}$ values were obtained by fitting the linear part of the curve. The slope of the curve gives the $K_{SV}$ value. The $a^*$ values were obtained from the modified Stern–Volmer plot (not shown) according to eqn 3.
inflection indicates a partially unfolded intermediate state, lack of an inflection does not necessarily exclude the existence of partially unfolded states. In the latter case, our results suggest that the intermediates are less stable. Differences in the stability of intermediate structures and the accessibility of tryptophans of ERα-E₂ and ERβ-E₂ complexes may represent availability of specific domains for interaction with accessory proteins and the potential for transcriptional activation of estrogen-responsive genes by ERα and ERβ.

A partially unfolded conformational state was not detectable by tryptophan fluorescence during the thermal unfolding of ERβ bound to high-affinity ligands such as E₂ or ERE. The single-step transition indicates higher thermodynamic stability of the complexes compared with ligand-free protein. Although E₂ does not change the T_M of ERβ, the labile nature of the intermediate state in the presence of E₂ shows significant conformational changes in its presence. Conversely, a stable intermediate structure in E₂-bound ERα represents a distinct conformational change compared with that of ERα. AFM revealed that the existence of compact ERβ particles after heating the samples to 37 °C, whereas ERα and ERβ showed undefined shapes and larger particles at 22 °C.

Previous studies of the unfolding of the LBD of ERα and ERβ (Gee & Katzenellenbogen 2001) using intrinsic tryptophan fluorescence in the presence of guanidine hydrochloride suggested the presence of a folding intermediate. The authors suggested that partial unfolding of the LBD provided an accessible hydrophobic interior, allowing ligand binding, dissociation, or exchange. Open and closed ligand-pocket conformations of ERα have also been described by studies of mutant ERs (Carlson et al. 1997). Our studies demonstrate conformational transitions and stabilization of intermediate structures in full-length ERα and ERβ in the presence and absence of E₂. The N-terminal (A/B) regions of ERα and ERβ are unstructured and this region is 80 amino acids shorter in ERβ than in ERα (Warnmark et al. 2001b, Kumar & Thompson 2003). Ligand binding and/or DNA binding may facilitate folded structures in the N-terminal region to different extent. The A/B region in turn may make contacts with LBD or DBD in functionally organizing the conformational states of ERα/ERβ. The differential effects of E₂ and ERE on ERα/ERβ may have contributions from the differences in the A/B region.

Studies on the ligand specificity of ERα and ERβ indicate that the overall difference in amino acid sequence of these proteins has resulted in LBD structures with unique ligand-binding affinities (Manas et al. 2004, Nettles et al. 2004). However, the functional similarities of ERα and ERβ do not dictate similar ligand-induced conformational changes in these proteins. Indeed the coactivator recruitment and binding affinities of ERα and ERβ are different (Hall et al. 2000, Warnmark et al. 2001a, Margeat et al. 2003). In addition, recent studies show that ERβ binds to the corepressor proteins in the presence of ER agonistic ligands such as E₂ (Webb et al. 2003). This is unusual among nuclear receptors as they generally bind corepressors in either the absence of ligands or the presence of antagonistic ligands (McKenna & O’Malley 2002).

The ratio and the level of ERα and ERβ vary in estrogen-responsive cells and tissues enriching the texture of their estrogenic responses. ERβ is the more expressed receptor in central nervous and cardiovascular systems, and prostate gland, whereas ERα plays a dominant role in mammary gland and uterus (Couse et al. 1997, Forster et al. 2004). ERα is believed to play a central role in the origin and progression of breast cancer; however, recent studies suggest that ERβ can suppress breast cancer progression (Paruthiyil et al. 2004, Strom et al. 2004). Thus ERα and ERβ appear to have different or even opposite biological actions on some tissues, although the mechanisms involved in these processes are not yet known.

Previous CD studies on the ligand-induced stabilization of ERα indicated that the presence of E₂ increased the T_M of ERα by 5 °C (Greenfield et al. 2001). Addition of ERE also increased the T_M of unfolding and the presence of E₂+ERE showed an additive effect. However, current studies using tryptophan fluorescence did not show a significant change in T_M due to the presence of E₂. However, the presence of ERE alone or E₂+ERE increased the T_M by 10 °C, as measured by tryptophan fluorescence. Although temperature-dependent quenching of tryptophan fluorescent as well as the CD signal at 222 nm represent unfolding of the protein, the sensitivity of these techniques to unfolding of ERα/ERβ might be different. Both techniques, however, detected large changes in the stability of ERα/ERβ induced by the presence of ERE.

AFM studies are often used to characterize intermediates formed during protein folding, unfolding, and aggregation. AFM imaging of γD crystallin during folding showed globular structures within the first minute of re-folding, aggregation and fibril formation by 24 min, and thick bundles by 1 h (Kosinski-Collins & King 2003). Spherical particles were also reported in the case of a mutant huntington fragment which was then converted to annular structures and fibrils in a time-dependent manner (Wacker et al. 2004). Our AFM studies provide confirmatory evidence of intermediate species observed by intrinsic tryptophan fluorescence and CD studies. In addition, AFM images show that the ERα intermediate is more prone to aggregate formation, whereas the ERβ intermediate exists as well-dispersed stable particles. Formation of compact intermediates, such as the molten globules and pre-molten globules, is an important step in amyloidogenesis and fibril
formation (Yon 2002, Uversky & Fink 2004). Although fibrils were not observed with ERα or ERβ, our studies demonstrate for the first time that full-length ERα and ERβ can form unfolding intermediates which have different tendencies to form aggregates.

In summary, our studies show unique conformational transitions in full length ERα and ERβ after their binding to E2 and the ERE. The ERE stabilizes both ERα and ERβ by forming a specific complex with increased ΔT_m (ΔT_m ~ 10 °C) compared with ligand-free ERs; however, control/mutant ODNs are unable to form such a stable complex. Ligand-free ERβ and E2-bound ERα formed stable intermediate states during temperature-dependent unfolding. AFM studies show the formation of highly compact particles of ERβ and a mixture of compact particles as well as less compact speckled structures for ERα. Fluorescence-quenching studies indicate that ERα and ERβ have two classes of tryptophans, one class being more buried than the other. The differences in the unfolding and quenching behavior of ERαE2 and ERβE2 complexes reveal differences in their conformational states. The ERαE2-ERE and ERβE2-ERE complexes also have different conformations as seen from their quenching behavior. Differences in the stability and structure of unfolding intermediates and the differential effects on quenching behavior in response to ligand binding might represent the functional differences between ERα and ERβ in coactivator recruitment and activation of transcription.

Acknowledgements

This work was supported by NIH grants CA42439, CA80163, and CA73058 from the National Cancer Institute, by ES05022 from the National Institute of Environmental Health Sciences (NIEHS Center Excellence), and by a grant from the Susan G. Komen Breast Cancer Foundation. We thank Sasha Chhabria for conducting some of the fluorescence measurements. Sasha Chhabria was supported by NIH grant CA42439S1. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Journal of Molecular Endocrinology (2005) 35, 211–223

Received in final form 16 July 2005

Accepted 27 July 2005

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