Altered Ligand Binding Properties and Enhanced Stability of a Constitutively Active Estrogen Receptor: Evidence That an Open Pocket Conformation Is Required for Ligand Interaction[†]

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ABSTRACT: To elucidate the ligand binding properties of the estrogen receptor (ER) and how ligand access to and release from the ligand binding pocket is affected by the conformational state of the receptor, we have measured the rates of estradiol association and dissociation, the equilibrium binding, and the stability of estradiol binding to denaturants, comparing wild-type human ER and a point mutant (Y537S ER) that shows full constitutive activity, i.e., the same full transcriptional activity in the absence or presence of estrogen. Ligand binding kinetics and affinity were measured with the full-length (1−595) ERs and with truncated forms of both receptors containing domains C through F (including the DNA binding, hinge, and ligand binding domains, amino acids 175−595) or domains E and F (the ligand binding domain; amino acids 304−595). With all ERs, the rates of ligand association and dissociation were considerably slower with the Y537S mutant ER than with wild-type ER (6-fold and 3−4-fold, respectively). These marked differences in ligand on and off rates for the wild-type and Y537S receptors result in a predicted (k_{-1}/k_{+1}) and measured K_d that is 2-fold lower for Y537S ER compared to wild-type ER. The binding of estradiol by wild-type ER was disrupted by high concentrations of urea (above 2 M), whereas the Y537S ER was distinctly more resistant to this disruption. These results are consistent with a model in which wild-type ER in the absence of ligand adopts a transcriptionally inactive collapsed pocket conformation, stabilized by specific interactions of Y537 with nearby regions of ER. When estradiol is bound, the wild-type ER adopts a transcriptionally active, closed pocket (ligand occupied) conformation. By contrast, the Y537S mutant ER favors the transcriptionally active closed pocket conformation, whether occupied by ligand or not, the latter state (closed pocket but unoccupied) accounting for its constitutive activity. Our findings suggest that the entry or exit of ligand from the binding pocket requires that ER adopt an open pocket conformation. The reduced rates of ligand association and dissociation in the constitutively active form of the ER, as well as its greater resistance to disruption of ligand binding by urea, support the supposition that the rate at which this open pocket conformation can be accessed from the unoccupied or ligand-occupied Y537S ER is slower than from the unoccupied or occupied forms of wild-type ER. Thus, the binding and release of ligand by ER require that the receptor access an open pocket state, and the ease with which this state can be accessed is affected by mutations that alter receptor conformation.

The estrogen receptor (ER),[1] a member of the nuclear hormone receptor superfamily, is a sequence-specific transcription factor which, like other sequence-specific transcription factors, has conserved DNA binding and transactivating domains (*1*−*4*). However, what distinguishes ER and many other members of the nuclear hormone receptor family from these other transcription factors is the fact that their activity is modulated by the binding of a small exogenous ligand (the hormone) to the ligand binding domain: In the absence of ligand, ER generally has little transcriptional activity, but the binding of an agonist ligand results in transcriptional activation. In ER and other nuclear receptors, ligand binding promotes the dissociation of heat shock proteins, strengthens receptor dimerization and DNA binding of the activated receptor at estrogen response elements in regulated genes, and enhances gene expression through receptor interaction with basal transcription factors, coactivators, and integrator proteins (*4*−*7*).

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[1] Abbreviations: BHA, butylated hydroxyanisole; E_2, estradiol; EDTA, ethylenediaminetetraacetic acid; ER, estrogen receptor; ESI-MS, electrospray ionization mass spectrometry; Gua·HCl, guanidine hydrochloride; HAP, hydroxylapatite; IPTG, isopropyl β-D-thiogalactoside; LBD, ligand binding domain; Ni-NTA, nickel-charged nitrilotriacetic acid; RA, retinoic acid; RARα, retinoic acid receptor-α; RXRα, retinoid X receptor-α; SDS, sodium dodecyl sulfate; SRC-1, steroid receptor coactivator-1; T_3, thyroid hormone; TR, thyroid hormone receptor; Tris, tris(hydroxymethyl)aminomethane; Y537S ER, estrogen receptor Tyr537Ser mutant.

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Numerous studies have indicated that ligand binding results in substantial alterations in the conformation of nuclear receptor ligand binding domains that can be detected through changes in receptor hydrophobicity, epitope exposure, protease sensitivity, and coregulator binding (*7*−*15*). Recent X-ray crystal structures of the ligand binding domains of representative members of the nuclear receptor class further support this view (*16*−*18*): In the complexes of the human retinoic acid receptor-γ (RARγ) with *all-trans*-retinoic acid (RA) (*16*) and of the rat thyroid hormone receptor-α1 (TR) with thyroid hormone (T_3) (*17*), the ligand is inside a "closed pocket," almost completely enveloped by structural elements that encompass about half of this domain of the receptor. By contrast, in the structure of an unliganded ligand binding domain (human retinoid X-receptor α; RXRα) (*18*), this portion of the domain has a very different, rather "collapsed pocket" conformation. Thus, the interactions between ligand and protein in these receptors appear to be detailed and multifaceted, and result in the remodeling of a significant portion of the surface topology of the receptor protein. This topological change is thought to alter the constellation of coregulator interactions that underlie the lack of transcriptional activity of the unliganded receptor vs the activity of the liganded one.

Considering the nearly complete envelopment of ligand by receptor seen in the RAR−RA and TR−T_3 complexes, it is not surprising that the rate of ligand *dissociation* from these closed complexes can be very slow. However, it is also of note that the rate of ligand *association* with nuclear hormone receptors is also very slow, being several orders of magnitude below the near-diffusion-controlled rates of ligand interaction with the more open active sites of enzymes, which are generally described as clefts. This suggests that substantial, and relatively slow, conformational changes in the receptor are needed for ligand to gain access to the binding pocket in unliganded receptors. If the rather collapsed pocket conformation of unliganded RXRα (*16*) is an accurate model for other empty nuclear hormone receptors, then a considerable loosening of portions of the receptor must occur for the binding pocket to open and for ligand to gain access to the pocket.

We and others have reported previously that, rather remarkably, some single point mutations in these nuclear hormone receptors can mimic the effect of the binding of agonist ligands, resulting in receptors that have partial or full constitutive transcriptional activity (*7*, *19*−*21*). These constitutively active mutant receptors appear to have conformations that—even when unoccupied by ligand—more resemble that of the liganded, transcriptionally active receptor in terms of sensitivity to proteases and the binding of coactivator proteins (*7*, *22*). Nevertheless, the constitutively active receptors still bind ligands with high affinity, with estradiol maintaining the high transcriptional activity seen in their ligand-unoccupied state and antiestrogens blocking their constitutive and estradiol-occupied transcriptional activity (*7*, *19*, *22*).

In this study, we have investigated the effect that a single point mutation in the ligand binding domain of the ER has on the ligand binding properties of ER and the resistance of receptor-bound ligand to disruption by denaturants. This Y537S mutation engenders strong constitutive activity, such that the Y537S ER shows nearly full constitutive activity, with its transcriptional activity in the absence of estrogen equal to that of the wild-type ER in the presence of maximally effective concentrations of estradiol (*7*). We have used full-length wild-type and constitutively active Y537S ERs, as well as shorter constructs of these receptors, to study the kinetics of ligand association and dissociation, the equilibrium binding affinity, and the stability of the wild-type and Y537S ERs toward the disruption of ligand binding by denaturants. Although the absolute rates and affinities of ligand binding differ, depending on the length of the expressed constructs, in all cases, the constitutively active Y537S mutant receptor shows a substantially slower rate of ligand association and a substantially slower rate of ligand dissociation. In addition, Y537S ER is more resistant to the disruption of ligand binding by urea treatment. These results support the idea that the Y537S mutation slows the rate of formation of the open pocket conformation of ER required for ligand to gain access to or escape from the binding pocket, implying that ligand binding (or activation of receptor by the Y537S mutation) results in a receptor having a distinctly different conformation of its ligand binding pocket.

MATERIALS AND METHODS

Materials. Radiolabeled estradiol ([^{3}H]E_2) ([6,7-^{3}H]estra-1,3,5,(10)-triene-3,17-β-diol), 51−53 Ci/mmol, rat Ig horseradish peroxidase linked whole antibody, Hybond nitrocellulose membrane, and the ECL kit were obtained from Amersham Corp (Arlington Heights, IL). Unlabeled estradiol, butylated hydroxyanisole (BHA), thrombin, isopropyl β-D-thiogalactopyranoside (IPTG), and yeast extract were from Sigma (St. Louis, MO); urea and guanidine hydrochloride from Fisher (Pittsburgh, PA). Hydroxylapatite (HAP; Bio-Rad, Hercules, CA) was prepared following the recommendations of Williams and Gorski (*23*). The pET15b vector was obtained from Novagen (Madison, WI); the competent BL21(DE3)pLysS *E. coli* cells were from Novagen (Madison, WI). Cell culture media and other reagents for cell culture were purchased from GIBCO BRL (Gaithersburg, MD). Calf serum was from Hyclone Laboratories (Logan, UT), and fetal calf serum was from Sigma. The H222 antibody was kindly provided by Dr. Geoffrey Greene (University of Chicago, Chicago, IL).

Estrogen Receptor Preparations. Full-length wild-type and Y537S estrogen receptors were expressed by transient transfection in COS cells, as described previously (*7*, *24*). The supernatant fraction of whole cell homogenates was used in the binding studies.

Constructs for the expression of ER binding domains in *E. coli* were prepared in a pET15b vector using standard methods. The 304−554 sequence from wild-type ER or the 304−595 and 175−595 sequences from both wild-type and Y537S mutant ERs were amplified from pCMV cDNA subclones of ER or Y537S ER, respectively, using 15 rounds of PCR (Expand High Fidelity PCR system; Boehringer Mannheim, Indianapolis, IN) with primers that added an N-terminal *Nde*I site and a C-terminal *Bam*HI site for insertion into the pET15b vector at these restriction sites. The forward primer added a GSS sequence after the Met and before the 6x-His tag. The DNA sequences of all plasmids were confirmed by dideoxy sequence analysis using a Sequenase 2.0 kit from Amersham (Arlington Heights, IL) or USB (Cleveland, OH). *E. coli* BL21(DE3)pLysS cells were transformed, grown at 37 °C, and induced with IPTG at 30 °C, according to standard protocols.

For receptor isolation and purification, the cell paste was suspended at 10 mL of buffer per gram of cell paste in 50 mM Tris buffer, pH 7.5, 10% glycerol, 0.1 mM butylated hydroxyanisole (BHA), and 10 mM mercaptoethanol. The resuspended cell paste was sonicated (Vibra cell sonicator with a micro probe; Sonic Materials, Inc., Danbury, CT) for 10 s at 60% power to shear the DNA. The cell debris and pellet fraction were separated from the supernatant by centrifugation for 30 min at 30000*g*. This supernatant could be used directly for the binding studies, or purified to near-homogeneity by batchwise or column adsorption onto a nickel-charged nitrilotriacetic acid−agarose resin (Ni-NTA−agarose; Qiagen Inc, Santa Clarita, CA), following standard protocols.

Electrospray ionization mass spectrometry (ESI-MS), according to methods we have described previously (*25*), was used to confirm the mass of selected purified ER preparations. Construct 7 (cf. Figure 1) showed a mass of 30 886 Da, which is very close to the mass of 30 888 Da expected for this construct with the N-terminal methionine removed by posttranslational processing. SDS−PAGE Western blot analysis of constructs 3−6 (cf. Figure 2) gave evidence of cleavage in the F-domain, as we have observed previously (*25*). This was confirmed by ESI-MS analysis of construct 6, which showed masses of 32 351 Da (29%) and 32 521 Da (71%), consistent with cleavage at 569 and 571 amino acids, as previously observed (*25*) (again for constructs having the N-terminal methionine removed by processing).

Western Analysis. Purified ER from the *E. coli* extracts (constructs 3−7 of Figure 1) and whole cell extracts of the ER transfected into COS cells (constructs 1−2 of Figure 1) were electrophoresed on a 10% Tricine−SDS−polyacrylamide gel (*26*). The purified ER was loaded at ∼0.1 μg per lane, and the COS cell ER was loaded to comparable intensity. Following electrophoresis, the proteins were electroblotted onto a nitrocellulose membrane and probed with the ER-specific primary antibody H222 at 0.1 μg/mL, incubated with horseradish peroxidase-conjugated secondary antibodies (SAR Mab-HPR at 1:1000 dilution), and detected by chemiluminescence with the ECL kit, following the manufacturer's recommendations.

Binding Experiments. For all binding experiments, the ER was diluted into binding buffer (50 mM Tris, pH 7.5, 10% glycerol, 0.1 mM BHA, 10 mM mercaptoethanol, 0.5% yeast extract). The bound ligand was assayed by adsorption onto hydroxylapatite for 15 min at 0 °C, followed by three washes with 1 mL of 0.05 M Tris, pH 7.3. After the last wash, the HAP pellet was resuspended in 0.5 mL of EtOH and counted in 4 mL of scintillation fluid. The addition of yeast extract (*27*) was needed to reduce the loss of receptor from very dilute solutions, by adsorption onto tubes and pipets.

Ligand Association Experiments. The ER was diluted to ∼2 nM in binding buffer, and [^{3}H]E_2 was added to give a final concentration of 30 nM. The samples were incubated at 25 °C, and aliquots were taken in duplicate from the incubation at various times within the first 10 min and then at longer times (45−90 min) for the measurement of maximum binding. A sample with the ER blocked by a 100-fold excess of unlabeled E_2 was included at the longer incubation times to measure background or nonspecific binding. At each time, aliquots were pipetted into a 10× volume of chilled 'stop' solution (300 nM unlabeled E_2 in 10 mM Tris, 1.5 mM EDTA, 3 mM NaN_3, pH 7.4). The quenched aliquots were kept on ice and assayed by adsorption onto HAP. The 'stop' solution was considered to be equivalent to one wash, so the samples were washed 2 additional times with HAP wash buffer. The nanomolar specific binding at each time point was linearized using the equation ln $[(B_{max} - B)/B_{max}]$ and plotted versus time. The pseudo-first-order association rate constants, determined from the slope of the above plot, were divided by the concentration of free estradiol to give the second-order association constant, expressed as nM^{-1} min^{-1}.

Ligand Dissociation Experiments. The ER was diluted to ∼1.5−2 nM in binding buffer. This solution was incubated with 5 nM [^{3}H]E_2 at 25 °C for 1 h. The sample was then divided into two aliquots, one for stability and one for dissociation. A blocked sample (see above) was included for stability only. Unlabeled E_2 was added to the dissociation sample to be 500 nM (100× over the [^{3}H]E_2); a proportional amount of buffer was added to the two stability samples, and the samples were incubated at 25 °C. Aliquots were taken from the dissociation experiment at 0, 0.5, 0.75, 1, 1.5, 2, and 4 h and from the stability experiments at 0, 1, and 4 h. At each time point, duplicate samples were removed from the incubation, and added to chilled microcentrifuge tubes which already contained 0.1 mL of HAP slurry. This was assayed as described above. The specific bound [^{3}H]-E_2 remaining in the dissociation samples was calculated as a percent of the stability samples to account for any loss of total binding activity, which amounted to 20−30% of the initial activity over the course of the experiment. The first order dissociation rate constant was obtained from the slope of the plot of ln *B* against time.

Equilibrium Ligand Binding: Scatchard Analysis. The protein was incubated with various concentrations of [^{3}H]-E_2 in the absence or presence of a 100-fold excess of unlabeled ligand for 1 h or longer at 25 °C. Aliquots of the incubation solution were used to determine the total [^{3}H]E_2 in the sample. The incubation solutions were then assayed by adsorption onto HAP. Data were processed according to the method of Scatchard (*28*).

Disruption of Ligand Binding by Denaturants. The receptor-containing preparation was diluted to 300 nM in ER and incubated at 0 or 25 °C with a minimum of 2 equiv of [^{3}H]E_2 for 3 h. The binding solution was then diluted 1:50 into various concentrations of urea or guanidine in buffer [0.01 M Tris, pH 7.4, 1.5 mM EDTA, 3 mM NaN_3 (w/v), 0.5% yeast extract (w/v)] and further incubated at the previous temperature for 2.5 h. The retained ligand was assayed by adsorption onto HAP, washed, and measured (for details, see above). At each concentration of denaturant, the concentration of bound [^{3}H]E_2 was expressed as a percent of that in an undenatured sample.

RESULTS

Expression, Purification, and Characterization of Estrogen Receptor Constructs. The seven estrogen receptor (ER) constructs that we have used in this study are shown in Figure 1. Full-length human wild-type ER and Y537S mutant ER were prepared by transient transfection of expression plasmids into COS cells (Figure 1, entries 1 and 2). Truncated wild-type and Y537S human ER domains C−F (entries 3 and 4) or E and F (entries 5 and 6), as well as wild-type ER

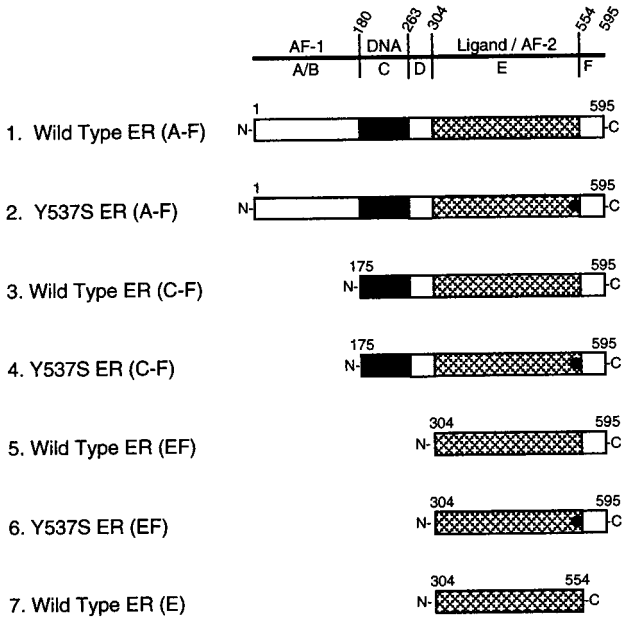

FIGURE 1: Estrogen receptor constructs used in this study. The domains of the estrogen receptor, denoted A−F, are shown schematically at the top of the figure. Full-length wild-type and constitutively active (Y537S) ERs (entries 1 and 2) were expressed in COS cells. Constructs 3−7 were expressed in *E. coli*. The smallest construct (entry 7) contains only the hormone binding domain, E. The Y537S mutation in constructs 2, 4, and 6 is shown as a boldface dot in domain E.

domain E alone (entry 7), were expressed in *E. coli* using a pET15b plasmid. This plasmid adds onto the expressed sequence of ER a 22 amino acid N-terminal extension that codes for a 6x histidine purification tag and a thrombin cleavage site. Under our bacterial expression conditions, a substantial fraction of the ER domains 3−7 was soluble and exhibited high-titer binding in the supernatant fraction from the bacterial lysate. These bacterially expressed receptors could be purified readily to near-homogeneity over a nickel resin, and the N-terminal tag removed by treatment with thrombin. Identical E_2 binding affinity and kinetics were observed for the bacterially expressed receptors, whether determined on the bacterial supernatant, or on the purified ERs with or without the N-terminal segment (data not shown). Therefore, the studies reported here were all performed on the bacterial supernatant without cleavage of the his-tag.

Western blots of the seven ER preparations are shown in Figure 2. Both the mutant Y537S and wild-type, full-length ER from COS cells appear as a 66 kDa doublet (lanes 1 and 2). Phosphorylation of ER can alter its electrophoretic mobility and cause it to appear as a doublet (*29*). The bacterially expressed constructs designed to include domain F (constructs 3−6) show size heterogeneity resulting from cleavage in the F-domain. This cleavage, at amino acids 569 and 571, was also seen in an earlier study (*25*) and was confirmed in construct 6 by electrospray ionization mass spectrometry (see Materials and Methods). This cleavage near the C-terminus in constructs 3−6 does not affect ligand binding affinity or kinetics, as shown by the identical behavior of constructs 5 and 7. Construct 7, that lacks the F domain, appears as a single species (Figure 2).

Rates of Ligand Association with Wild-Type and Y537S Estrogen Receptor Constructs. We have determined the association rates of [^{3}H]estradiol ([^{3}H]E_2) with these receptor

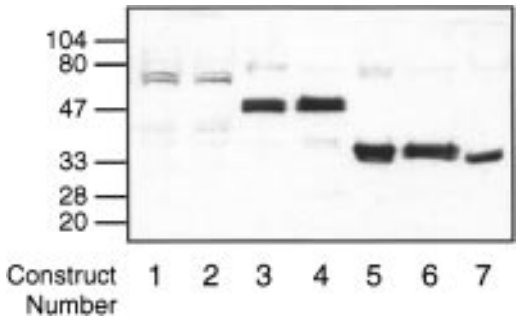

FIGURE 2: Western blot of the ER proteins used in this study. The full-length (1−595) ERs (constructs 1 and 2), expressed in COS cells, appear as 66 kDa species. Constructs 3 and 4 (175−595) and 5 and 6 (304−595), expressed in *E. coli*, show size heterogeneity expected from partial cleavage at the 569 and 571 sites. The *E. coli* expressed 304−554 construct (construct 7) is homogeneous.

preparations at 25 °C. The rates were measured using a 15-fold excess of ligand over receptor, so that pseudo-first-order kinetics could be assumed. The association rates (k_{+1})are summarized in Table 1 and Figure 3, and representative plots of the time course of association (cf. Figure 4) show that the fit of the association curves to a pseudo-first-order model is very good. This was true in each case.

With all three pairs of ER constructs (304−595, 175−595, and full-length 1−595 ER from COS cells), E_2 association with mutant ER was consistently *ca.* 6 times slower than with the comparable wild-type construct. Figure 4 shows the association data, from a typical experiment, with the pair of 304−595 constructs. In this experiment, the constitutively active mutant ER (Y537S) had an association rate constant nearly 8 times slower than the wild-type. The absolute rates of E_2 association with both ER forms depended on the length of the constructs, being somewhat faster with the shorter constructs (Figure 3 and Table 1).

Rate of Ligand Dissociation with Wild-Type and Y537S Estrogen Receptor Constructs. Dissociation rates (k_{-1}) were measured by an exchange assay in which an excess of unlabeled estradiol was added to an ER preparation that had been saturated by preincubation with [^{3}H]E_2 for 1 h at 25 °C. The dissociation rates are summarized in Table 1 and Figure 3, and representative plots of the time course of dissociation (construct 304−595, Figure 1, entries 5 and 6) are shown in Figure 5. The ligand dissociation process was cleanly first order over at least 70−80% of the dissociation process (cf. Figure 5). In all cases, the Y537S mutant had a 3−4-fold slower dissociation rate for estradiol than did the corresponding wild-type construct (Figure 5). As was true with the association rates, the dissociation rates were slower with the longer receptor constructs. A similar effect of receptor length on the rate of ligand dissociation from the androgen receptor has been reported (*30*).

Equilibrium Binding of Estradiol to Wild-Type and Y537S Estrogen Receptor Constructs. The equilibrium binding of [^{3}H]E_2 to the ER preparations was assayed by conventional saturation titration methods. Most incubation times were for 1 h, with the exception that longer than typical incubation times (5 h) were used in some cases because of the very slow ligand association with the Y537S ER preparations; ligand association would not have been complete after the standard 1 h incubation. K_d values are given in Figure 3 and Table 1, and representative Scatchard plots (construct 304−595, Figure 1, entries 5 and 6) are shown in Figure 6.

Table 1: Affinity and Kinetics of Estradiol Binding to Wild-Type and Mutant Y537S ER Constructs[a]

	ER construct		association,[c] k_{+1}	dissociation[d] k_{-1}	predicted K_d,[e]	
	domains	amino acids[b]	(nM^{-1} min^{-1}) (*n*)	(min^{-1}) (*n*)	(k_{-1}/k_{+1}) (nM)	Scatchard[e] K_d (nM) (*n*)
1.	A−F wt	1−595	0.030 ± 0.003 (2)	0.0158 ± 0.0082 (2)	0.53	0.617 ± 0.093 (2)
2.	(A−F) Y537S	1−595	0.0051 ± 0.0008 (2)	0.0059 ± 0.0006 (2)	1.16	0.705 ± 0.141 (2)
	[wild type/mutant]		*[5.9]*	*[2.7]*	*[2.2]*	*[1.1]*
3.	C−F wt	175−595	0.038 ± 0.012 (2)	0.027 ± 0.0036 (2)	0.71	0.732 ± 0.084 (3)
4.	(C−F) Y537S	175−595	0.006 ± 0.002 (2)	0.007 ± 0.0028 (2)	1.17	1.371 ± 0.213 (5)
	[wild type/mutant]		*[6.4]*	*[3.9]*	*[1.6]*	*[1.9]*
5.	EF wt	304−595	0.072 ± 0.023 (2)	0.045 ± 0.012 (5)	0.63	0.605 ± 0.141 (2)
6.	(EF) Y537S	304−595	0.011 ± 0.0001 (2)	0.015 ± 0.005 (5)	1.36	1.411 ± 0.171 (2)
	[wild type/mutant]		*[6.5]*	*[3.0]*	*[2.2]*	*[2.3]*
7.	E wt	304−554	0.089 ± 0.027 (2)	0.042 ± 0.004 (4)	0.472	0.680 ± 0.0085 (2)

[a] All assays were performed at 25 °C. Binding was assayed by the HAP adsorption procedure as detailed under Materials and Methods. [b] Constructs 3−6 are partially truncated at residues 569 and 571 (see text). [c] The association rates were determined with a 15-fold excess of ligand over receptor, so that pseudo-first-order kinetics can be assumed. [d] The samples for dissociation rate determinations were preincubated at 25 °C for 1 h; excess unlabeled estradiol was then added, and the dissociation was allowed to proceed at 25 °C. [e] The K_d in column 5 is predicted from k_{-1}/k_{+1}, whereas the K_d in column 6 is the experimental value obtained from the Scatchard plots after incubation at 25 °C for 1 h or 5 h; see Materials and Methods.

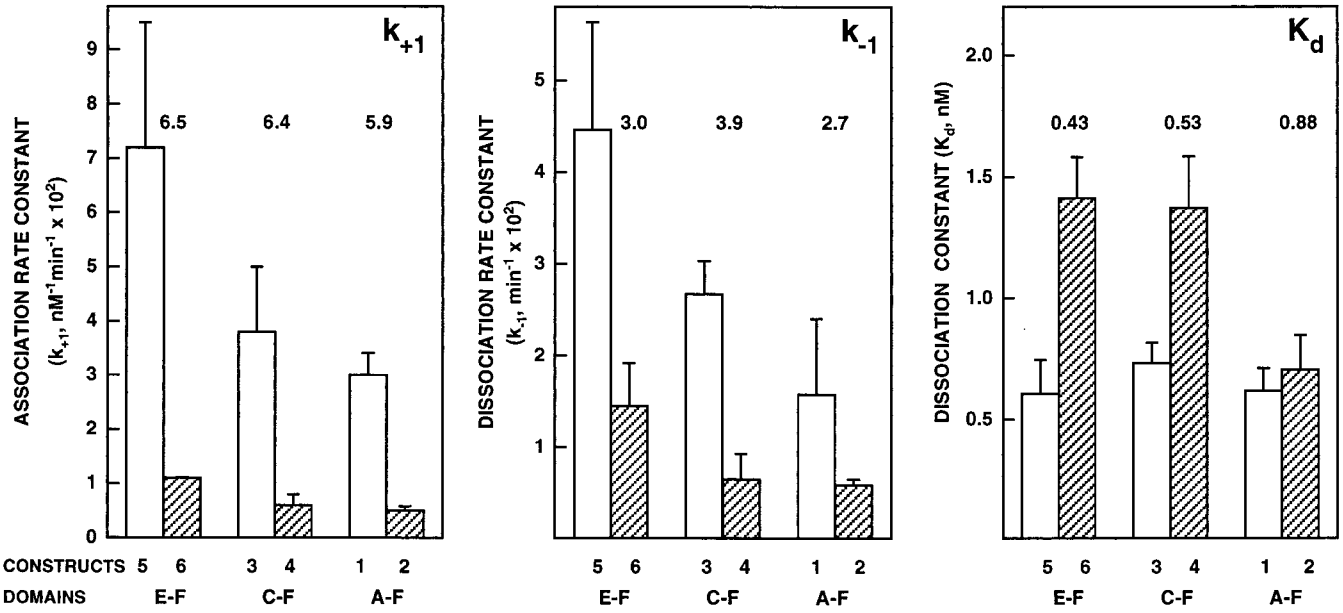

FIGURE 3: Graphical summary of the rates of estradiol (E_2) association (k_{+1}), dissociation (k_{-1}), and equilibrium binding (K_d) to the seven ER constructs. Data from wild-type ER are shown as open bars, and from the corresponding Y537S mutants as shaded bars. Values represent the average of multiple independent determinations ($n = 2-5$), and the error bars represent the range ($n = 2$) or standard error ($n > 2$). The ratio of the rates or the equilibrium binding constants between wild-type and the corresponding Y537S mutant are given above each pair of bars.

The Y537S mutant ER had a 2−3-fold lower affinity than wild-type ER.

The K_d of each construct can be predicted from the ratio of dissociation to association rate constants, k_{-1}/k_{+1}. Using this formula, the mutant Y537S ER is predicted to have a slightly higher K_d than the wild-type ER, and indeed this is observed (Table 1). Also, as shown in Table 1, the predicted and measured K_d values are quite similar.

Sensitivity of Estrogen Receptor Ligand Retention to Denaturants. The capacity of wild-type ER and Y537S ER to retain bound E_2 in the face of increasing concentrations of the denaturants guanidine hydrochloride (Gua·HCl) or urea at 0 °C or 25 °C is shown in Figure 7. The more potent denaturant Gua·HCl promoted ligand dissociation at relatively low concentrations (0.5−1.0 M), with little apparent difference between the wild-type and mutant ER. However, much higher concentrations of urea (2−4 M) were required to promote E_2 dissociation from the Y537S ER, and at both 0 °C and 25 °C temperatures, the Y537S mutant appeared distinctly more resistant to the disruptive effects of the denaturant. At both temperatures, ca. 0.5 M higher urea concentrations were needed to effect an equivalent degree of ligand binding disruption with Y537S ER compared to wild-type ER.

DISCUSSION

The Constitutively Active Y537S Estrogen Receptor Shows Slower Ligand Binding Kinetics and Greater Stability of Ligand Binding Than Does Wild-Type Estrogen Receptor. The major findings we present in this report are that the constitutively active Y537S ER mutant shows significantly slower rates of estradiol (E_2) association and dissociation than does wild-type ER, and that the mutant ER is also more resistant to disruption of E_2 binding caused by urea. The differences in ligand binding kinetics, which amount to an average 6-fold reduction in ligand association rate and a 3−4-fold reduction in ligand dissociation rate, are evident both in full-length ER as well as in shorter constructs that include only domains C through F or E through F. The absolute rates of ligand association and dissociation become progres-

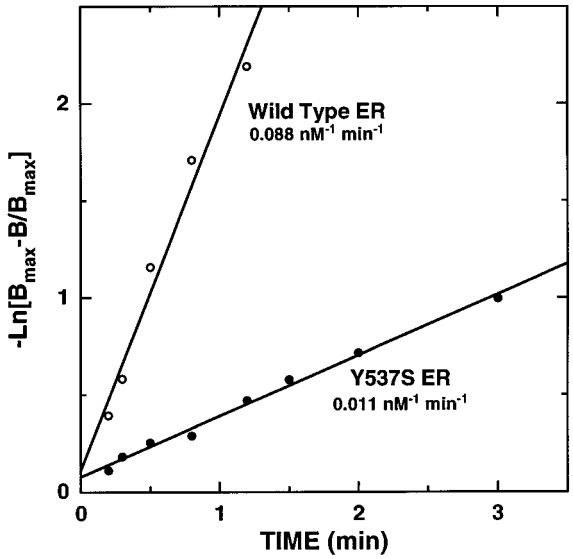

FIGURE 4: Ligand association kinetics of the Y537S mutant (construct 6) and wild-type ER (construct 5). This representative assay, at 25 °C, shows the rate of E_2 association with mutant ER to be *ca.* 8-fold slower than to wild-type ER.

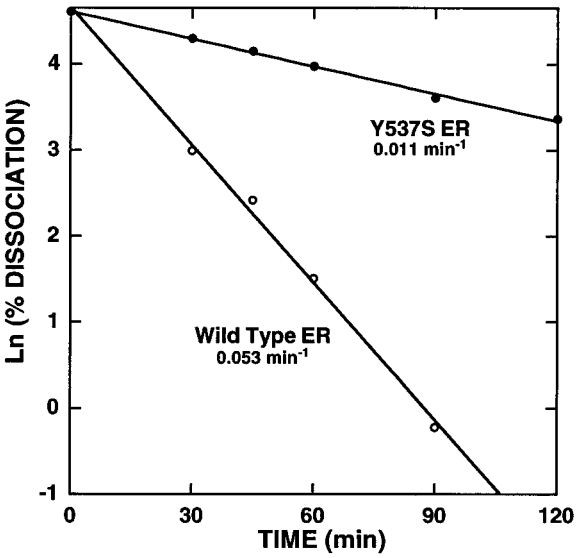

FIGURE 5: Ligand dissociation kinetics of the Y537S mutant ER (construct 6) and wild-type ER (construct 5). The ERs were prebound with [^{3}H]E_2 for 1 h at 25 °C; excess unlabeled E_2 was added, and the rate of dissociation was measured. In this experiment, the rate of E_2 dissociation from the mutant ER was 5-fold slower than from the corresponding wild-type construct.

sively faster with the smaller length constructs, while the ratio between wild-type ER and Y537S ER constructs remains the same.

The effect of urea in promoting ligand dissociation provides another avenue for assessing the stability of the ER−E_2 complex. The greater stability of this complex with the Y537S mutant was evident from the higher urea concentrations required to disrupt binding. It may seem contradictory that Y537S ER, which binds E_2 with a somewhat lower affinity than does wild-type ER, is able to retain bound E_2 in the face of higher urea concentrations. However, because ligand binding in the urea disruption assay is being measured under nonequilibrium (dissociating) conditions, this experiment in effect assays the degree to which ER is able to retain prebound ligand, i.e., the degree to which ligand dissociation rate is sensitive to urea.

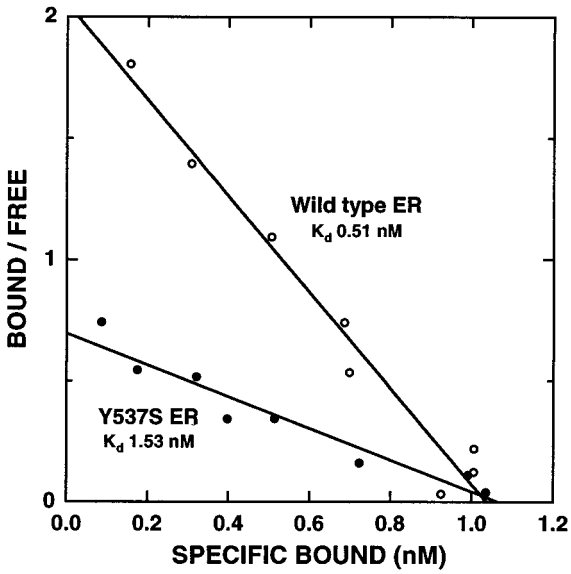

FIGURE 6: Scatchard plots of ligand binding to the 304−595 mutant ER (construct 6) and wild-type ER (construct 5). These assays were incubated for 1 h at 25 °C. With this construct and these conditions, the association of E_2 to this Y537S construct had a $t_{1/2}$ of 9 min, so the association was complete within 1 h (see text). Wild-type ER has a 3-fold higher affinity for estradiol than does the mutant.

Altered Ligand Binding Characteristics of the Constitutively Active Estrogen Receptor Suggest a Dynamic Model for the Ligand Binding Properties of the Estrogen Receptor. Tyrosine 537 in wild-type ER is an important site that is conserved in all known ER sequences, including the recently described ER subtype ERβ (*31*, *32*). It has been identified as a site of receptor phosphorylation and, along with other phosphorylation sites, has been implicated in the non-ligand-induced regulation of ER activity by growth factor signaling pathways (*33*−*36*). Tyrosine 537 phosphorylation has also been suggested to affect ligand binding, receptor dimerization, and transactivation activity (*37*−*39*). Studies from our laboratory (*7*), as well as recent studies of others (*19*, *20*), have demonstrated that certain Y537 mutants have moderate to very high constitutive activity. A significant finding from these reports is that substitution of Y537 with residues that cannot be phosphorylated (e.g., alanine, phenylalanine, asparagine) generates receptors that have high transcriptional activity and still bind estradiol with good affinity, making it unlikely that Y537 phosphorylation per se plays a significant role in regulating ligand binding and transcriptional activity. Those Y537 mutants that exhibit high ligand-independent, constitutive activity appear, as well, to manifest several characteristics of ligand-occupied wild-type ER: In the absence of ligand, they interact with receptor coactivator proteins such as SRC-1, and they show protease digestion patterns identical to those of the E_2-occupied ER and different from unoccupied ER (*7*, *22*). Thus, ER mutations at tyrosine 537 that confer constitutive activity cause ER to adopt a conformation even in the absence of ligand that resembles that of an ER complexed with an estrogen agent (and that is presumably different from that of a transcriptionally inactive ER complex with an estrogen antagonist).

Our findings, that the potent constitutively active Y537S ER also shows markedly reduced rates of ligand association and dissociation and greater resistance of ligand binding to disruption by urea, suggest that a new, dynamic dimension needs to be added to a working model for receptor

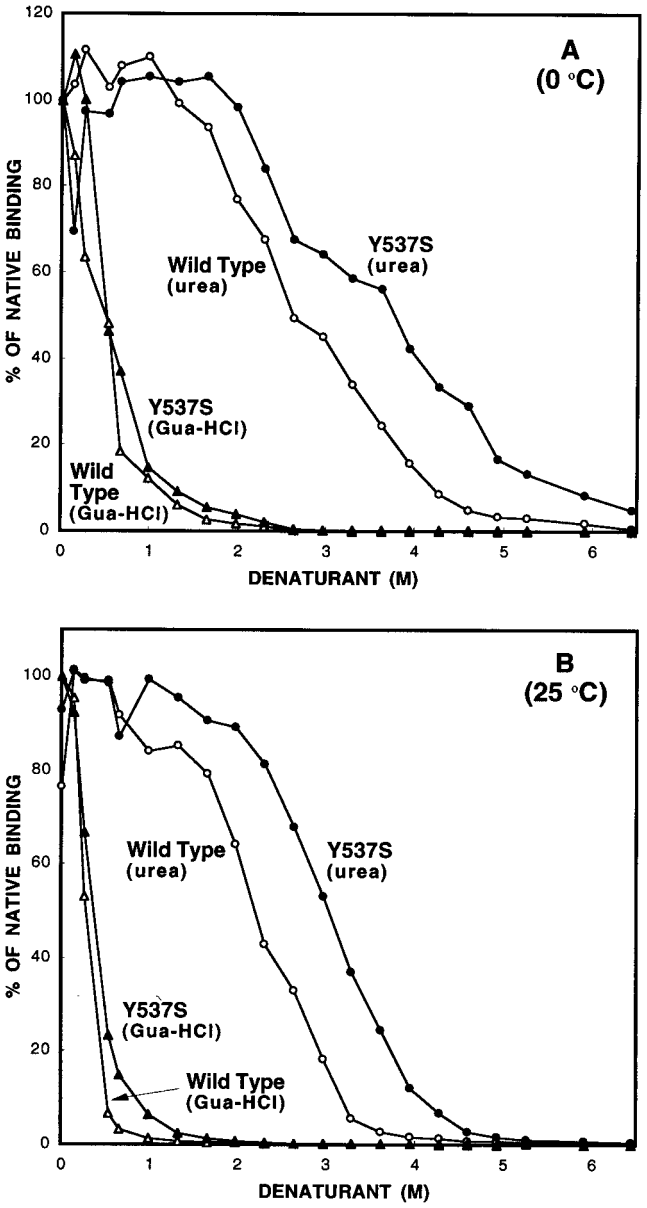

FIGURE 7: Urea disruption of estradiol retention by the 304−595 mutant ER (construct 6) and wild-type ER (construct 5) was determined by a standard ligand binding assay after the ER−E_2 complex had been equilibrated for 2.5 h with the indicated concentration of urea or guanidine hydrochloride (Gua·HCl), either at 0 °C (panel A) or at 25 °C (panel B).

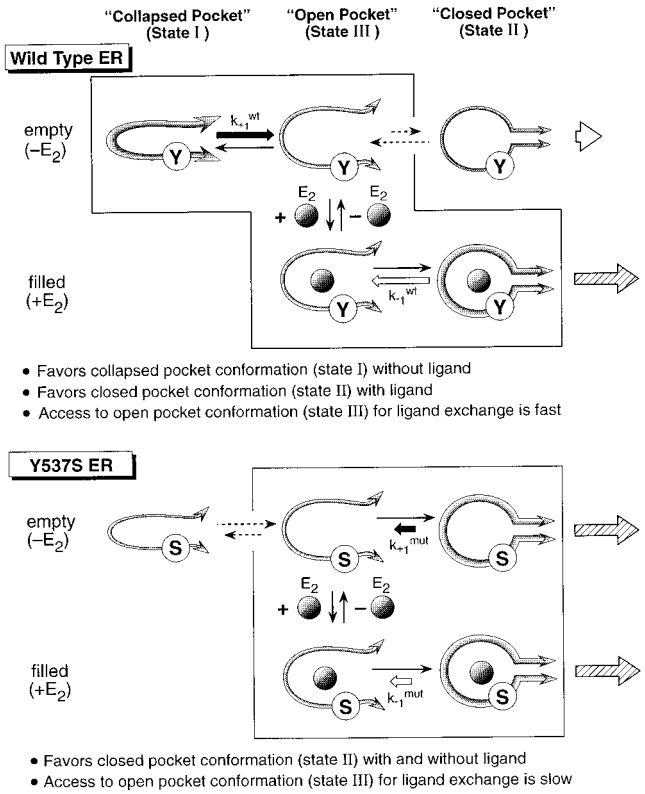

FIGURE 8: Three-state schematic model for ER, illustrating the process of conformational reorganization involved in ligand association and dissociation in wild-type ER (top) and Y537S ER (bottom). The collapsed pocket conformation (state I, left) is unoccupied and is transcriptionally inactive. The closed pocket conformation (state II, right) is transcriptionally active whether it is ligand-filled or empty. The open pocket conformation (state III, middle) is transient, but must be accessed for ligand to associate with or dissociate from the other forms. The two principal conformations for wild-type ER and Y537S ER are shown in boldface, and the principal routes of conformational isomerism are shown within the boxed region. The relative rates of isomerization between the various states of wild-type and Y537S ER are illustrated by the relative lengths of the arrows connecting the states. The transformations that determine the ligand association rates are indicated with bold black arrows; those that determine the ligand dissociation rates are indicated as thick, open arrows. The residue at position 537 (Y or S) is shown in the small circle. The magnitude of receptor transcriptional activity is illustrated by the large shaded arrow at the right of the figures. The ligand (E_2) is illustrated by a shaded circle.

conformation, ligand binding, and transcriptional activity of these nuclear receptors. This model, presented in Figure 8, can account for the four characteristics that the Y537S mutation confers on ER: (a) ligand-independent transcriptional activity, (b) a ligand-bound conformation in the absence of ligand, (c) reduced rates of both ligand association and dissociation, and (d) greater resistance to urea-induced ligand release.

Two Conformational States for Wild-Type Estrogen Receptor, Collapsed-Pocket (Unoccupied) and Closed-Pocket (Occupied), Are Presumed from Nuclear Receptor X-ray Structures. X-ray structures are currently published for only three nuclear receptor family members: retinoid X receptor-α, retinoic acid receptor-γ, and thyroid hormone receptor (*16*−*18*). Comparison of the X-ray structures of the nuclear hormone receptors without ligand (RXRα) and with ligand (RARγ−RA and TR−T_3) suggests that about half of the receptor molecule adopts a markedly different conformation upon ligand binding. Specifically, in the absence of ligand [as illustrated by the apo-RXRα structure (*18*)], the receptor is in the form of a "*collapsed pocket*" conformation (state I), with the void normally occupied by the ligand being largely filled by the inward movement of the β-strands and helix-11; in this form, the C-terminal activation function-2 (AF-2) activation helix (helix 12) is displayed in an outward orientation. By contrast, when a ligand is bound [as illustrated by the holo receptor structures of RAR and TR (*16, 17*)], these same three segments of the receptor are reoriented to form a "*closed pocket*" conformation (state II) that surrounds the ligand.

In homology models of ER based on these crystal structures, tyrosine 537, in the unliganded, collapsed pocket conformation (state I), would lie within the binding pocket in a *helical region* that is present between helices 11 and 12, whereas Y537 in the ligand-bound conformation is found at the extreme C-terminal end of the *loop* that forms between helices 11 and 12. Thus, this residue is well positioned to

affect the ligand-induced conformational reorganization of the receptor. White et al. (*19*) suggest that the presence of tyrosine at this position stabilizes the collapsed, transcriptionally inactive conformation of ER by interaction with nearby portions of the receptor, thereby minimizing ER basal activity. Conversely, replacement of tyrosine at this position with other residues less capable of this specific stabilization would allow ER to adopt a transcriptionally active conformation even in the absence of ligand, giving rise to constitutive activity.

Interestingly, a similar effect is documented for specific mutations in certain G-protein-coupled receptors: The large third cytoplasmic loop in these seven transmembrane structures is thought to play an inhibitory role in the unliganded receptor, blocking association with G-proteins; mutations in this loop that interfere with this role result in receptors with high basal, constitutive activity (*40*−*42*).

A Third, Open Conformational State for the Estrogen Receptor Is Needed To Account for the Altered Ligand Binding Characteristics and Enhanced Resistance to Disruption by Denaturants of the Y537S Mutant Receptor. Whereas the "collapsed" and "closed" conformations of ER (states I and II) can account for the transcriptionally inactive and active states of the receptor, these static, limiting structures do not address the issue of how ligand gains access to the collapsed form or escapes from the closed form. Divergent models for ligand access have been proposed for RAR−RA (involving the opening of helix 12 from the closed conformation) (*16*) and for TR−T_3 (involving movement of the long ω-loop between helices 1 and 3) (*17*). Regardless of the details of this process, however, it is evident that for ligand to escape from the closed conformation, a portion of the receptor has to open up. Conversely, for ligand to gain access to the collapsed conformation, this form of the receptor also needs to adopt a more open conformation (state III). Thus, ligand exchange in ER is proposed to occur from a third "open pocket" conformation. *The retarding effect that the Y537S mutation has on the rates of ligand association and dissociation, and the greater resistance to urea-induced ligand dissociation, suggests that this mutation has perturbed the rates at which the "open pocket" conformation can be accessed from the other two states.* Figure 8 illustrates a schematic model for these three states of ER and their interconversion for wild-type and Y537S ER.

In wild-type ER (Y537) (Figure 8, top), the receptor exists in the collapsed pocket conformation (state I) in the absence of ligand; this form is transcriptionally inactive and is stabilized by specific interactions between Y537 and nearby residues in this collapsed conformation. Addition of E_2, which occurs via the open pocket conformation (state III), promotes formation of the E_2-occupied state, i.e., closed pocket−occupied conformation (state II), which is transcriptionally active; the interactions of E_2 with ER thus overcome the stabilizing effect that Y537 has in the collapsed pocket state. By contrast, in the Y537S mutant (Figure 8, bottom), the specific stabilization of the collapsed pocket form by Y537 no longer occurs, so the transcriptionally active closed pocket form is favored both in the presence and in the absence of E_2, the latter situation leading to the constitutive activity of Y537S ER.

What is significant in terms of the rates of ligand association and dissociation is the following: (a) the reduced rate of ligand association means that the rate at which the open-pocket state (III) forms from the closed pocket−unoccupied form of Y537S ER is slower than from the collapsed pocket state (I) of wild-type ER (compare k_{+1}^{mut} vs k_{+1}^{wt}, boldface arrows); (b) the reduced rate of ligand dissociation means that the rate at which the open pocket state (III) forms from the closed pocket−occupied state (II) is slower with Y537S ER than wild-type (compare k_{-1}^{mut} vs k_{-1}^{wt}; open arrows).

It is not surprising that there is a difference in ligand association rates between the wild-type and Y537S ERs, because the open pocket conformation (state III) is being accessed from two very different unoccupied conformations of receptor, the open pocket conformation (state I) for wild-type vs the closed pocket conformation (state II) for the Y537S form. The closed pocket conformation (favored for unoccupied Y537S ER) may simply be more stable than the collapsed pocket conformation (favored for unoccupied wild-type ER) and thus slower to open. Reasons for the slower rate of ligand dissociation from the occupied mutant Y537S vs wild-type ER are less obvious, because the open state is being accessed from similar closed pocket conformations (state II) for both receptors. From the difference in ligand dissociation rates, one must surmise that substitution of tyrosine 537 by serine makes the Y537S ER−E_2 complex more reluctant to open up, a fact that is also supported by its greater resistance to the effect of high concentrations of urea.

It is interesting to speculate on the nature of the open-pocket conformation of ER (state III) that is suggested by these ligand binding kinetic experiments. It is clearly less stable than the collapsed or closed states, and is therefore of only transient existence. It is also possible that the open pocket conformation does not, in fact, have a discrete structure. Rather, it may be a form of ER in which a portion of the ligand binding domain is incompletely folded and has the expanded and more dynamic tertiary structure expected for the molten globule state of proteins. Further experiments to characterize the open pocket state of ER are underway. Regardless of the specific nature of this open-pocket state, our results indicate that the binding and release of ligand proceed through this state and that the rate at which this state can be accessed is affected by mutations that alter receptor conformation.

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