Accelerated Publications

Conformational Changes in Chicken Thyroid Hormone Receptor α1 Induced by Binding to Ligand or to DNA†

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ABSTRACT: A classic model of steroid/thyroid hormone receptor activation postulates that a conformational change or "transformation" occurs upon ligand binding as a first step toward regulation of gene transcription. In order to test this model, physical studies have been carried out using purified full-length chicken thyroid hormone receptor α1 (cT_3R-α1) expressed in *Escherichia coli*. Circular dichroism spectroscopic studies reveal that cT_3R-α1 adopts a different conformation upon specific binding to a cognate ligand triiodothyroacetic acid as well as to a thyroid hormone response element, an idealized inverted repeat AGGTCA TGACCT. These results suggest that cT_3R-α1 may adopt distinct conformations whether free or bound to ligand or to DNA. These states may reflect the changes in the conformation of steroid/thyroid hormone receptors in the signal transduction pathway.

The thyroid hormones regulate the growth, development, and metabolism of essentially all tissues of higher organisms (Samuels et al., 1988) in part via binding to a nuclear receptor protein (Samuels & Tsai, 1973). The thyroid hormone receptor (T_3R) is a member of a superfamily of ligand-responsive transcription factors (Evans, 1988). The steroid/thyroid hormone receptors are related by several well-defined domains which confer such functions as DNA binding, transcriptional activation, dimerization, and ligand binding. These receptors can be classified according to DNA sequence recognition (Umesono & Evans, 1989). The ligand binding domain consists of more than 200 amino acids and is the least conserved domain within the members of the steroid/thyroid hormone receptors. As expected, ligand binding specificity is strict among this superfamily (Forman & Samuels, 1990). Upon binding to ligand, the receptor is thought to undergo a conformational change which mediates transcription of hormone-responsive genes.

A major unanswered question is the molecular mechanism of the interaction between steroid/thyroid hormone receptors and their cognate ligands and DNA recognition elements. To date, precise structural information is available only on the DNA binding domain of the glucocorticoid (Härd et al., 1990; Luisi et al., 1991) and estrogen (Schwabe et al., 1990) receptors, which constitutes less than ~10% of the intact protein. While this information is useful toward an understanding of the nature of DNA binding domain peptide/DNA contacts, no information is available with regards to receptor–ligand binding. Furthermore, despite much information concerning the physiological roles and the molecular biology of this family of receptors, no physical data have been reported on the intact proteins. Unlike human T_3R-β1 expressed as insoluble inclusion bodies (Lin et al., 1990), chicken T_3R-α1 (cT_3R-α1) and human retinoic acid receptor (Forman et al., 1992) are the only full length steroid/thyroid hormone receptors that can be expressed in soluble and functional form in *Escherichia coli* and do not appear to require accessory factors to bind ligand or DNA (Picard et al., 1990).

To explore both the secondary structure and the potential influence of ligand and DNA on receptor structure of cT_3R-α1, we have employed circular dichroism spectroscopy. These studies reveal that cT_3R-α1 adopts distinct conformations whether free or bound to ligand or to DNA, each of which may play roles in the mediation of transcriptional events.

MATERIALS AND METHODS

Purification of cT_3R-α1. cT_3R-α1 was purified from lysates of *E. coli* BL21 DE3 pLYSs which express receptor from pET-cT_3R-α1 polyA⁻ (Forman et al., 1992). After induction by 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), bacteria were lysed by a freeze/thaw cycle in 25 mM Tris, pH 7.8, 0.5 mM Na_2EDTA, 400 mM KCl, 15% glycerol, 0.05% Triton X-100, 1 mM PMSF, and 10 mM β-mercaptoethanol (GTME-400). The receptor was purified to homogeneity as described (Forman et al., 1992) by sequential ammonium sulfate fractionation, polyethyleneimine precipitation, heparin agarose chromatography, and size exclusion chromatography with a final yield of ~1 mg of pure receptor per liter of bacterial culture (see Table I). Samples were resolved using a Mini-PROTEAN II 12% polyacrylamide gel according to the manufacturer's protocol (Bio-Rad). Proteins were then visualized using Coomassie blue dye. A final purity of >90% was assessed by scanning a polyacrylamide gel loaded with

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various quantities of receptor using an LKB Ultrascan XL Enhanced Laser Densitometer. Staining intensity was found to be linear between 1.0 and 5.0 μg of receptor.

T_3 Binding Assays. Binding reactions were carried out in 500 μL as described (Samuels et al., 1974) using 100 fmol of receptor and 2 nM L-3,5,3′-[^{125}I]triiodothyronine [^{125}I]T_3 (2200 Ci/mmol, New England Nuclear, NEX-110X) with the indicated concentration of unlabeled ligand (Figure 1). Nonspecific binding was measured using parallel reactions containing 1 μM unlabeled T_3 and represented 10–15% of the total receptor-bound [^{125}I]T_3. Receptor-bound [^{125}I]T_3 was separated from the free [^{125}I]T_3 using 2.5-mL columns of Sephadex G25-80 (fine, Sigma) as described previously (Samuels et al., 1974).

Electrophoretic Mobility Shift Assays. Various dilutions of purified cT$_3$R-α1 were incubated with ~5 fmol (30 000 cpm) of ^{32}P-labeled idealized inverted repeat thyroid response element (TRE$_{ir}$) and resolved using a 4% nondenaturing polyacrylamide gel (Forman et al., 1992). Reactions were carried out in the absence of hormone. TRE$_{ir}$ contains the inverted repeat 5′ AGGTCA TGACCT 3′ flanked by *Hin*dIII overhangs 5′ AGCTT 3′ and is blunt-end labeled with [α-^{32}P]-dTTP using the Klenow fragment of DNA polymerase. No binding was detected to a mutant TRE (TRE$_m$) containing 5′ ACGTCA TGACGT 3′ (vide infra, circular dichroism studies). The equilibrium constant for dissociation (K_d) for the receptor–DNA complex was estimated using the intensities of free and bound DNA as detected on the autoradiogram.

Oligonucleotide Synthesis and Purification. Oligonucleotides were synthesized on a 0.2-μmol scale and purified using a Milligen oligonucleotide purification column followed by separation on a C_4 reverse-phase HPLC column with a 50 mM triethylammonium acetate/acetonitrile gradient. Complementary strands were annealed in 10 mM triethylammonium bicarbonate buffer on a micromolar scale by heating at 95 °C for 10–15 min and cooling slowly to room temperature. The annealed oligonucleotide was dialyzed against GTME-400 buffer and then concentrated to 50–60 μM using a Millipore Ultrafree-MC filter (10 000 molecular weight cutoff). The purity of the samples was checked by labeling with T4 polynucleotide kinase and [γ-^{32}P]ATP. Samples employed in the circular dichroism studies were predominantly double-stranded oligonucleotide.

Circular Dichroism (CD) Spectroscopy. CD spectra were acquired using a Jasco J-720 spectropolarimeter. Each spectrum was averaged over four scans, corrected for signal from buffer, smoothed, and then converted to mean residue ellipticities. Protein was quantitated using the Bio-Rad protein microassay using bovine serum albumin as a standard. Samples were placed in a thermally jacketed cylindrical quartz cuvet of 0.1-mm path length. High buffer absorbance in the far UV produced an unacceptably poor signal/noise ratio in the 180–190-nm region.

Thermal Denaturation Studies. Thermal denaturation of cT$_3$R-α1 was measured by monitoring CD at 222 nm. An AVIV 62 DS CD spectrometer, equipped with a thermoelectric temperature control device, was used. Samples were placed in a stoppered quartz cuvet of 1-mm path length, and the temperature was scanned at a constant rate of 2 °C intervals. An equilibration time of 1 min was allowed at each temperature, and the signal was averaged for 30 s. Ellipticity at 260 nm (nearly zero at 4 °C) was also collected simultaneously as a function of temperature, and this curve was subtracted from the ϕ_{222nm} versus temperature plot to eliminate nonspecific background effects. The midpoint of the thermal transition (T_m) was estimated using the first derivative of the thermal denaturation curve.

Table I: Purification of Chicken T$_3$R-α1[a]

purification step	specific binding[b]	total protein (mg)	fold purification
crude lysate	70	150	1.00
PI	14	150	0.20
AS1 supernatant	19	48	0.27
AS2 cut	34	16.6	0.48
heparin agarose	2060	3.44	29.4
FPLC	20 000–30 000	1.19	290–430

[a] Typical data for an 800-mL preparation using pET-cT$_3$R-α1 polyA$^-$. Crude lysate is estimated to contain ~0.33–0.8% cT$_3$R-α1 by binding to [^{125}I]T_3. Final purity was assessed to be > ~90% by laser densitometry of a Coomassie Blue stained SDS–polyacrylamide gel. PI, polyethyleneimine; AS1, ammonium sulfate 25% saturation; AS2, ammonium sulfate 35% saturation; FPLC, Superose 12HR 10/30 (Pharmacia). [b] Picomoles of bound [^{125}I]T_3/mg of protein.

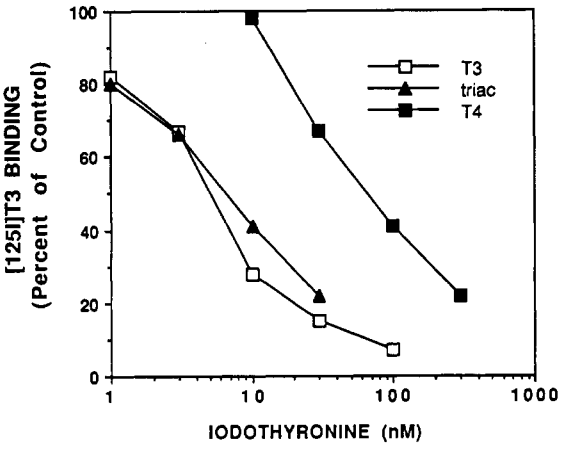

FIGURE 1: Relative affinity of T_3, triac, and T_4 for purified cT$_3$R-α1. Binding reactions were carried out as described under Materials and Methods.

RESULTS

Using a pET-8c vector (Rosenberg, 1987; Studier et al., 1990) to express the cT$_3$R-α1 cDNA, the receptor accumulates to approximately 0.3–1% of the total soluble protein in crude *E. coli* lysate (Table I) based on specific binding of L-3,5,3′-[^{125}I]triiodothyronine ([^{125}I]T_3). Purified cT$_3$R-α1 binds [^{125}I]T_3 with an equilibrium dissociation constant K_d of ~1 nM (Forman et al., 1992). A relative binding study shows that the purified receptor has similar affinities for T_3 and 3,5,3′-triiodothyroacetic acid (triac), a 10-fold lower affinity for L-thyroxine (T_4) (Figure 1), and a 100-fold lower affinity for L-T_2 (data not shown). These relative affinities parallel the relative biological activity of these ligands (Samuels et al., 1988) and are similar to those reported for T$_3$Rs synthesized in reticulocyte lysates (Sap et al., 1986; Munoz et al., 1988) or present in the nuclei of eucaryotic cells (Samuels et al., 1974; Schueler et al., 1990). A large fraction of the purified recombinant cT$_3$R-α1 binds hormone since the amounts of receptor estimated by protein assay or specific [^{125}I]T_3 binding were similar assuming that one molecule of receptor binds one molecule of T_3.

DNA binding activity of cT$_3$R-α1 was assessed by measuring specific binding to the TRE$_{ir}$ (Figure 2) in the absence of hormone. Two gel shift complexes were observed which have been previously identified as receptor monomers and dimers (Forman et al., 1992). The purified receptor does not bind to a similar DNA sequence containing two G to C changes

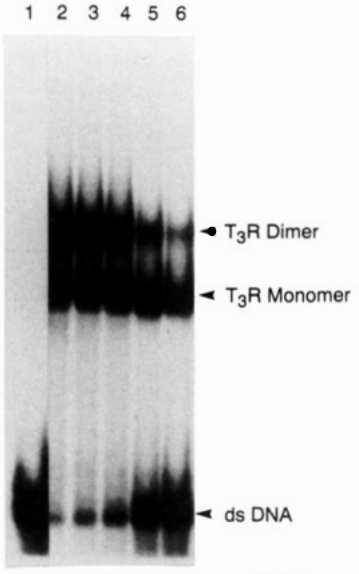

FIGURE 2: Purified cT_3R-$\alpha 1$ binds to a TRE-containing oligonucleotide (TRE_{ir}). (Lane 1) No receptor, (lane 2) 14.5 pmol (~670 ng) of cT_3R-$\alpha 1$, (lane 3) 3.63 pmol, (lane 4) 1.45 pmol, (lane 5) 0.363 pmol, (lane 6) 0.145 pmol.

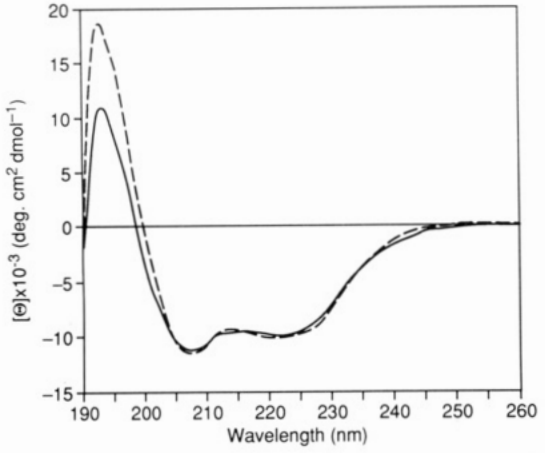

FIGURE 3: CD spectra of cT_3R-$\alpha 1$ (10 μM) in the absence (—) and presence of 10 equivalents triac (– – –) at 5 °C. The reaction conditions employed were identical to those described for binding to [^{125}I]T_3, except that free ligand was not removed.

(TRE_m) (ACGTCA TGACGT) (vide infra). A K_d of cT_3R-$\alpha 1$ binding to the TRE_{ir} is estimated as (0.4–1) × 10^{-8} M.

Circular dichroism (CD) spectroscopy was employed to define the secondary structure content of cT_3R-$\alpha 1$ under conditions in which the receptor is active with regards to ligand and DNA binding. Because of the highly absorbing nature of the constituents of the solution (400 mM KCl, 0.05% Triton X-100, 15% glycerol, and 10 mM β-mercaptoethanol) that were necessary to maintain active receptor, measurements could not be made below 190 nm. The CD spectrum of cT_3R-$\alpha 1$ exhibits a strong maximum at 193 nm and minima at 208 and 222 nm (Figure 3). Secondary structure analysis was carried out using the PROSEC method obtained from AVIV associates (Chang et al., 1978). The approximate fractional contributions of α-helix, β-sheet, turns, and unordered structures were estimated at 0.28 ± 0.01, 0.28 ± 0.08, 0.11 ± 0.02, and 0.33 ± 0.06, respectively. These estimates are derived using averaged data from four separate samples. The fraction of helical content of the receptor is significantly lower than the predicted values obtained using either the automated Chou-Fasman (Chou & Fasman, 1974) or Garnier algorithms (Garnier et al., 1978) of 0.50 and 0.45, respectively.

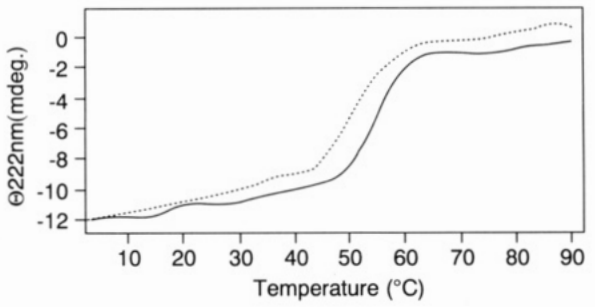

FIGURE 4: Thermal denaturation of cT_3R-$\alpha 1$ (10 μM) in the absence (- - -) and presence (—) of 2.3 equiv of triac as monitored by CD at 222 nm.

The role of ligand on the conformation of cT_3R-$\alpha 1$ was then investigated using CD spectroscopy. Because of its higher solubility, triac was used instead of T_3 to study the effect of ligand on receptor conformation. Addition of triac to cT_3R-$\alpha 1$ leads to an increase in the magnitude of ellipticity around the 193 nm maximum of the receptor CD spectrum (Figure 3). The mean residue ellipticity values at 193 nm were 11 390, 17 520, and 18 710 deg·cm^2·dmol^{-1} in the presence of 0, 1-, and 10-fold excess of triac. The ellipticities at the 208- and 222-nm bands were not altered beyond the range of experimental error. Triac also produced a small but reproducible increase in the stability of the receptor conformation against thermal denaturation as monitored by CD at 222 nm. The T_m for cT_3R-$\alpha 1$ was raised from 50 to 54 °C upon addition of a 2.3-fold molar excess of triac (Figure 4), corresponding to a stabilization of ~1.6 kcal/mol (Branden & Tooze, 1991).

The interaction of cT_3R-$\alpha 1$ with the TRE_{ir} 22 base pair oligonucleotide in the absence of ligand was also accompanied by a significant increase in ellipticity around 193 nm over and above the CD spectra of the free receptor and oligonucleotide samples (Figure 5A). The observed change in the CD spectrum is more dramatic than that observed using receptor in the presence of triac (Figure 3). However, there was no change in the near-UV spectrum (260–350 nm) of the oligonucleotide upon interaction with cT_3R-$\alpha 1$, suggesting that the induced far-UV spectral change around 193 nm reflects a conformational change in the receptor and not the DNA. Addition of up to a 4-fold molar excess of TRE_m oligonucleotide, which does not bind to cT_3R-$\alpha 1$, did not produce any appreciable change in the CD spectrum of the receptor (Figure 5B). Interestingly, similar experiments carried out using up to a 4-fold molar excess of an oligonucleotide containing a TRE half-site ($TRE_{1/2}$) (AGGTCA TGACGT) or a TRE direct repeat with a four-nucleotide gap (TRE_{dr+4}) (AGGTCA acgt AGGTCA) led to little or no change in the CD spectrum of the receptor (data not shown), even though T_3Rs can bind to these DNA elements (Forman et al., 1992; Umesono et al., 1991).

DISCUSSION

Addition of the hormone T_3 to T_3Rs has been shown to alter the chromatographic mobility of the T_3R–T_3 complex (Silva et al., 1977) as well as the electrophoretic mobility of the receptor–TRE_{ir} oligonucleotide complex (Forman et al., 1992). This suggests that the receptor undergoes a change in conformation upon binding to ligand or to DNA. Whether DNA alters the conformation of receptor in the absence of ligand cannot be assessed by electrophoretic mobility shift experiments. The present studies address for the first time the secondary structure elements of cT_3R-$\alpha 1$ as estimated using CD spectroscopy in the absence and presence of triac,

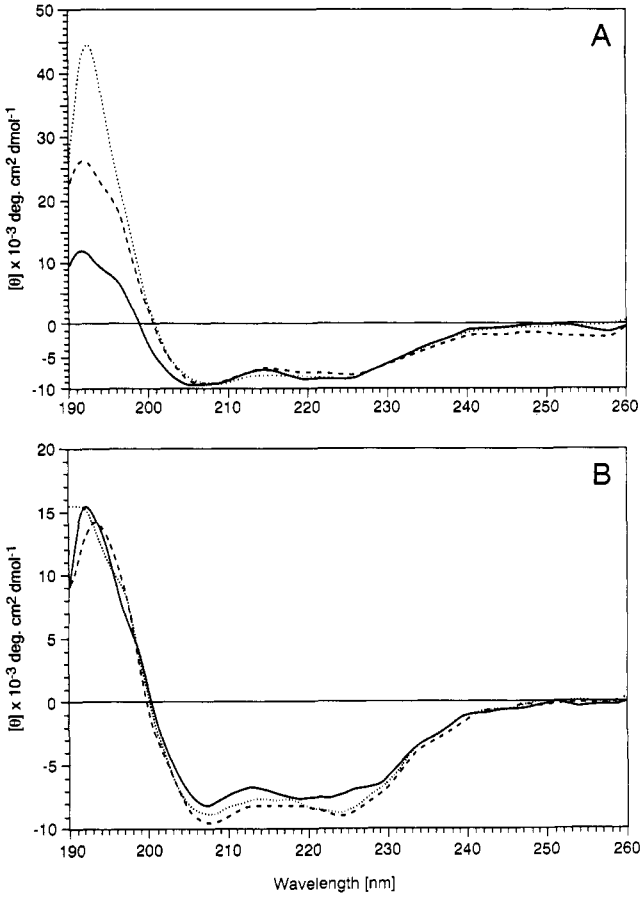

FIGURE 5: CD spectra of cT_3R-α1 (5 μM) in GTME-400 as a function of added TRE_{ir} (A) or TRE_m (B). (—) 0.0, (- - -) 1.0, and (···) 4.0 equiv of oligonucleotide. Spectra are corrected for buffer and for oligonucleotide.

a T_3 analog, as well as in the presence of several DNA recognition elements. Addition of a molar excess of triac led to an increase in ellipticity at 193 nm. One interpretation of the induced CD around 193 nm is a subtle structural change of the receptor induced by ligand binding, such as small alterations of the angles of rotation around the N–C_α bond phi (ϕ) and the C_α–C′ bond psi (ψ) within the protein without a major perturbation of the secondary structure (Manning & Woody, 1991). Another possible explanation is altered far-UV contribution of aromatic residues in the receptor upon binding to triac. The alternative possibility that this enhanced CD is a reflection of receptor-induced CD in triac itself cannot be ruled out at present. However, free triac does not exhibit a significant CD signal in the far UV.

The interaction between cT_3R-α1 and the TRE_{ir} oligonucleotide was accompanied by a dramatic increase in ellipticity at 193 nm with no appreciable change in the near-UV spectrum due to oligonucleotide alone. This suggests that a conformational change is induced in the receptor and not the DNA and is consistent with the recently reported crystal structure of the glucocorticoid DNA binding domain complexed with a 19 base pair oligonucleotide (Luisi et al., 1991) in which the DNA adopts an unperturbed B-form helix. Solution spectroscopic studies of the yeast transcriptional activator GCN4 DNA binding domain complexed to a 21 base pair AP-1 site oligonucleotide also support this idea (Weiss et al., 1990).

No measurable change in the CD spectrum was observed upon addition of a molar excess of either the TRE_m, $TRE_{1/2}$, or the TRE_{dr+4} oligonucleotides. This suggests that the perturbation in the CD spectrum of cT_3R-α1 in the presence of TRE_{ir} is specific to the interaction between the receptor and the inverted repeat (IR) response element. This finding may reflect the mode of receptor interaction(s) with these different elements (Forman et al., 1992). The TRE_m does not bind receptor while the $TRE_{1/2}$ only binds the receptor as a monomer and not as a dimer (Forman et al., 1992). The TRE_m and $TRE_{1/2}$ also exhibit virtually no biological activity (Forman et al., 1992). The TRE_{dr+4} is a functional response element and can bind receptor (Umesono et al., 1991). The IR elements, however, tend to be more biologically active than the direct repeat (DR) response elements with identical half-sites (Forman et al., 1992). Furthermore, the receptor would be expected to form different contacts in the carboxy-terminal dimerization domain when binding to an IR element (head to head) than to a DR element (head to tail) (Forman & Samuels, 1990; Forman et al., 1992). These differences could lead to different protein conformations which may not be detectable using circular dichroism depending upon the time scale of the interaction and on conformational averaging. Further physical chemical studies are needed to understand the nature of the interaction of the receptor with the $TRE_{1/2}$ and in particular the TRE_{dr+4} element.

It is of interest to compare the crystal structure of the glucocorticoid DNA binding domain bound to DNA to the NMR solution structure of the DNA binding domain itself (Härd et al., 1990). Upon binding to DNA, the DNA binding domain peptide undergoes hydrogen-bonding interactions within a segment of an antiparallel β-sheet in the amino-terminal "zinc finger" as well as a distortion within the α-helix in the second "zinc finger". These subtle structural changes are consistent with the observed change in the CD spectrum of intact cT_3R-α1 upon binding to a TRE_{ir} oligonucleotide. However, it is not known whether similar structural changes observed with the glucocorticoid DNA binding domain peptide upon binding to DNA also occur with the intact glucocorticoid receptor.

These data represent the first physical study using a full-length protein as a representative of the steroid/thyroid receptor superfamily. This approach may lead to an understanding of the role of ligand and of DNA binding in altering the conformation of the receptor to a state that is capable of mediating activation of transcription.

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