Structure of the Hormone Binding Domain of Human β1 Thyroid Hormone Nuclear Receptor: Is It an α/β Barrel?

Peter McPhie,[‡] Clifford Parkison,[§] B. K. Lee,[§] and Sheue-yann Cheng*,[§]

Laboratory of Biochemistry and Metabolism, National Institute of Diabetes and Digestive and Kidney Diseases, and Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

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ABSTRACT: To understand the structure of the hormone binding domain (HBD) of human β1 thyroid hormone nuclear receptor (h-TRβ1), truncated h-TRβ1 fragments, MD32 (M^{169}–D^{456}), KD29 (K^{201}–D^{456}), DD28 (D^{211}–D^{456}), KD25 (K^{235}–D^{456}), and KP28 (K^{201}–P^{448}), were analyzed by circular dichorism (CD). MD32 and KD29 show intense CD spectra with double minima at 222 and 208-210 nm, indicating the presence of extensive regions of α-helix. DD28 and KD25 have spectra which are reduced in intensity with minima around 215 nm, characteristic of a β-sheet. The observed spectra are compatible with sequence analysis which predicts that HBD contains alternating stretches of α-helix and β-strand. These extensive decreases in secondary structure in DD28 and KP28 in which the predicted first β-strand or last α-helix was deleted, respectively, were accompanied by the loss of hormone binding activity. On the basis of these results, we suggest a new model for h-TRβ1 consisting of the known DNA binding domain linked by an α-helical hinge to the HBD, with the tertiary structure of an α/β barrel. The model is compatible with previous chemical and genetic studies on the structure of this protein.

The thyroid hormone, 3,3′,5-triiodo-L-thyronine (T_3),[1] has diverse effects. It promotes growth, induces differentiation, and regulates metabolic and developmental processes. Studies on the mechanism of T_3 action have been facilitated by the isolation of cDNAs for thyroid hormone nuclear receptors (TRs). There are two major TR isoforms, TRα and TRβ, whose genes are located on chromosomes 17 and 3, respectively (Oppenheimer, 1991). Recent studies have indicated that TRs bind to specific DNA sequences known as thyroid hormone response elements (TRE). Binding of TRs with TREs of target genes leads to gene activation. The interaction of TR with TRE is modulated by other nuclear proteins, including the retinoid X receptor (Marks et al., 1992; Zhang et al., 1992; Leid et al., 1992; Yu et al., 1991).

Thyroid hormone receptors are members of the steroid/retinoic acid/vitamin D receptor superfamily. Their transcriptional activity is dependent on thyroid hormone. From sequence homologies with other receptors, human β1 thyroid hormone receptor (h-TRβ1) could be assigned four functional domains: A/B (M^1–L^{101}), C (C^{102}–A^{170}), D (T^{171}–K^{237}), and E (R^{238}–D^{456}) (Green & Chambon, 1986). The amino-terminal A/B domains are thought to be involved in activation and repression of target genes. Domain C, the only region of the molecule whose structure is understood, contains two zinc fingers. The first of these binds DNA while the second has been shown to participate in protein–protein interactions (Luisi et al., 1991). These are linked by domain D to domain E, which alone was thought to bind thyroid hormone (Evans, 1988). The mechanism of hormone-dependent gene regulation will become much clearer when the structure of the entire receptor is determined.

Using a series of deletion mutants, we recently showed that both domain D and the eight carboxyl-terminal residues of domain E are essential for hormone binding activity (Lin et al., 1991). In addition, recent studies also demonstrated that the carboxyl-terminal sequence of h-TRβ1 is critical for its association with RXRs (Zhang, 1992). To understand the molecular basis of the critical role of these regions in the function of the hormone binding domain (HBD), we studied the secondary structures of a series of truncated h-TRβ1 mutants by circular dichroism (CD) and now propose a tertiary structure for the HBD of h-TRβ1.

MATERIALS AND METHODS

[3′-^{125}I]T_3 (2200 Ci/mmol, 1 Ci = 37 GBq) was purchased from DuPont-New England Nuclear. Isopropyl β-D-thiogalactopyranoside was from Research Organics, Inc. (Cleveland, OH). Triton X-100, aprotinin, phenylmethanesulfonyl fluoride, and leupeptin were from Sigma (St. Louis, MO).

Purification of the Hormone Binding Domain of h-TRβ1 and Its Truncated Proteins. The truncated proteins shown in Figure 1A were purified from *Escherichia coli*. Expression and isolation of the inclusion bodies were carried out similarly as described previously (Lin et al., 1991). All subsequent steps were carried out at 4 °C. The inclusion bodies were resuspended by brief sonication (5 s) in 1/100 of the original cell volume of 5 M guanidine hydrochloride 50 mM Tris·HCl (pH 7.8), and 5 mM EDTA. After clarification, the supernatant was added dropwise to cold, rapidly stirring buffer containing 50 mM Tris·HCl (pH 8.0), 20% glycerol, 1 mM DTT, 10 μg/mL aprotinin, 1 μg/mL leupeptin, and 0.1 mM phenylmethanesulfonyl fluoride. After being stirred gently for 20 h, the solution was clarified (85000*g* for 30 min) and dialyzed against 50 mM Tris·HCl (pH 8.0) and 50 mM NaCl for 3 h with at least two changes of buffer. Once dialyzed, the protein was loaded onto a Mono Q anion exchange column (Pharmacia-LKB, Uppsala, Sweden) in 50 mM Tris·HCl (pH 8.0) and 50 mM NaCl, and the column was then washed with the same buffer. The column was eluted using a 20-column vol of salt gradient from 50 to 500 mM NaCl in 50 mM

* Author to whom correspondence should be addressed: Bldg. 37, Rm. 4B09, National Cancer Institute, Bethesda, MD 20892. Telephone: (301) 496-42890. Fax: (301) 402-1344.

‡ National Institute of Diabetes and Digestive and Kidney Diseases.

§ National Cancer Institute.

[1] Abbreviations: T_3, 3,3′,5-triiodo-L-thyronine; TR, thyroid hormone nuclear receptor; h-TRβ1, human β1 TR; TRE, thyroid hormone response element; HBD, thyroid hormone binding domain; CD, circular dichroism; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

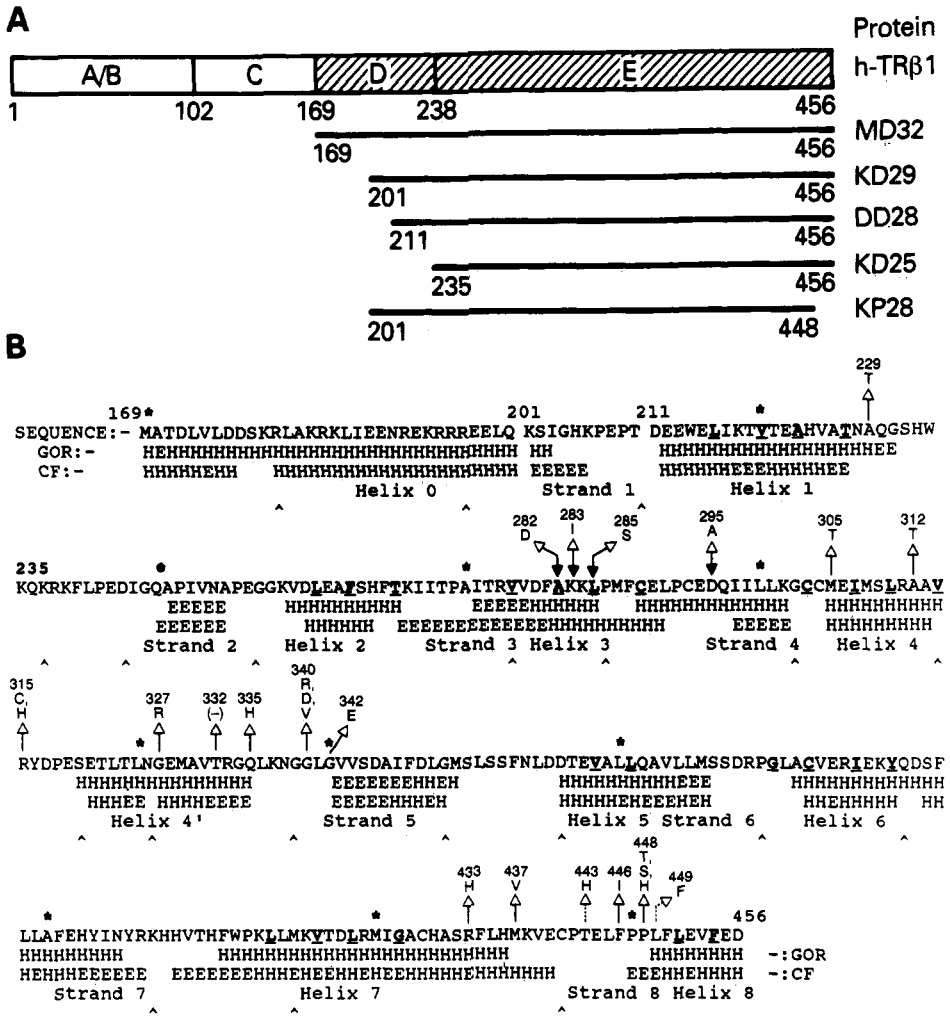

FIGURE 1: (A) Schematic representation of the deletion mutants of h-TRβ1. Domains were assigned according to Green and Chambon (1986). (B) Secondary structural predictions for domains D and E of h-TRβ1. The approximate locations of the predicted structural elements are shown in the bottom lines. Residues are predicted to be helical (H) or in an extended sheet (E); an asterisk indicates maxima in hydrophobic plot; a caret indicates maxima in Flexplot. Heptad repeats of hydrophobic residues are indicated by bold-face underlined residues. Mutations identified in the β1 gene of the affected kindred of thyroid hormone resistance (Refetoff, 1992) are also shown. The numbers refer to the positions of amino acid residues according to Weinberger et al. (1986). Deletion is indicated by –. An open, solid arrow indicates point mutation. An open, dotted arrow indicates frame shift mutation. An arrow with two ends, one open one dark, indicates point mutations resulting in the impairment of transcriptional activity of TR (O'Donnel et al., 1991).

Tris·HCl (pH 8.0) using an FPLC system (Pharmacia-LKB, Uppsala, Sweden). The eluted protein fractions were evaluated by SDS–PAGE analysis and T_3 binding assays. The fractions of interest were pooled and run over a G3000SW TSK sizing column (TSO-HAUS, Philadelphia, PA) using 50 mM Tris·HCl (pH 8.0) and 0.2 M NaCl. The purified protein fractions of interest were pooled, and protein concentrations were determined by amino acid analyses.

Binding of T_3 to the Purified Proteins. The binding was carried out by incubating the proteins purified from above with 0.2 nM [^{125}I]T_3 in the presence or absence of unlabeled T_3 in 0.25 mL of buffer B (50 nM Tris·HCl (pH 8.0), 0.2 M NaCl, 0.01% Lubrol, and 20% glycerol) for 90 min at 25 °C. Protein-bound [^{125}I]T_3 was separated from the unbound radioligand in a Sephadex G-25 (fine) column (5.5 × 1 cm), as described previously (Lin et al., 1991).

Circular Dichroism (CD) Spectra. CD spectra were recorded at 25 °C, in a Jasco J-500C spectropolarimeter, using a DP-500N data processor and 1 mm path length quartz cuvettes in 0.1 M sodium phosphate buffer. Spectra were digitized and downloaded as before (McPhie & Shrager, 1992) and analyzed in terms of secondary structure by least-squares fits using the PC-MLAB computer program (Civilized Software Inc., Bethesda, MD). Protein concentrations (50–100 μg/mL) were measured by amino acid analysis. Solutions of proteins were held in a cell holder, which was thermostated by a Lauda circulating water bath. Single wavelength thermal melting curves were measured as described previously (McPhie & Shrager, 1992).

For the inhomogeneous carboxyl-terminal mutant proteins, CD spectra were measured for three column fractions containing varying proportions of KP28 and KP28-L (see Figures 1A and 2B). The relative amounts of the two proteins in each fraction were estimated densitometrically after their separation by SDS–PAGE. The experimental spectra were then resolved into the intrinsic CD spectra of KP28 and KP28-L by matrix analysis, using PC-MLAB.

Sequence Analysis. Predictions were performed and averaged on the entire sequences of nine related thyroid hormone receptors (see Discussion), but only the relevant domains D and E of h-TRβ1 are shown (Figure 1B). Secondary structure predictions were made using the programs of Garnier et al. (1978) and Chou and Fasman (1978). Only predicted elements longer than four residues (one turn of an α-helix) are shown. Hydrophobic plots and chain flexibilities were calculated using the Hydropathy program of Kyte and

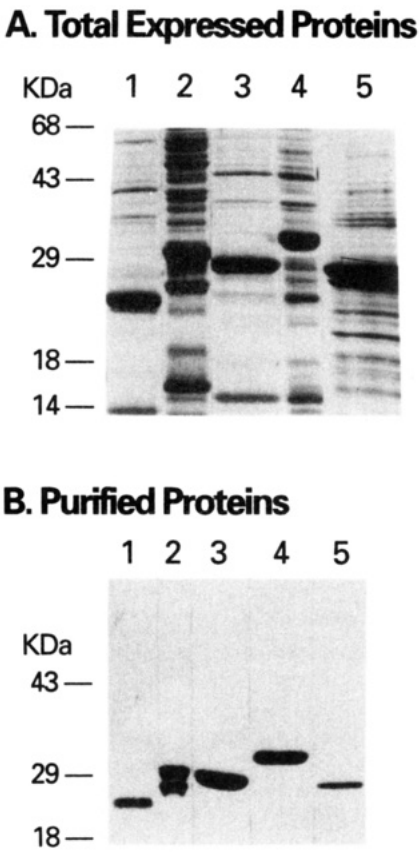

FIGURE 2: Total expressed proteins in *E. coli* and the purified proteins analyzed by SDS–PAGE. The total *E. coli* lysate (~0.1 mL cell suspension) (A) and the purified proteins (2–4 μg) (B) were loaded onto 4–20% and 12% polyacrylamide gels, respectively. After electrophoresis, gels were stained with Coomassie blue. The order in lanes 1–5 is KD25, KP28, KD29, MD32, and DD28.

Doolittle (1982) and the Flexplot program of Karplus and Schulz (1985), respectively. Where the secondary structure prediction methods disagreed, the structure was assigned by consideration of local hydrophobicity patterns. Helices were limited by previously detected (Forman et al., 1989) heptad repeats of hydrophobic residues (underlined bold-face residues in Figure 1). Sheets were assigned to local maxima in the hydrophobic plot and loops to local maxima in the Flexplot.

RESULTS

Purification of the Hormone Binding Domain of h-TRβ1 and Its Truncated Proteins. The truncated proteins of h-TRβ1 were expressed in *E. coli* using expression vectors which contain a T7 promoter, the Shine-Dalgarno sequence, and the coding sequence for the truncated proteins shown in Figure 1A. The proteins were highly expressed in *E. coli* as shown in Figure 2A. The proteins were purified using protocols modified from those previously reported (Lin et al., 1991). Figure 2B shows the purified proteins. All proteins showed one single band in an over-loaded SDS–PAGE, except KP28 which copurified with a protein with an apparent molecular weight of 30 000 (KP28-L, lane 2 in Figure 2A,B). Copurification of KP28 and KP29-L through multiple chromatographic steps suggests a close similarity in their chemical and physical characteristics. To understand the relationship between KP28 and KP28-L, we determined the amino-terminal sequences; both gave the same amino-terminal sequence of KSIGHKPEP. This sequence is the same as that derived from a cDNA sequence (Weinberger et al., 1986). We also used an anti-peptide antibody C-91 which recognizes the carboxyl-terminal sequence of 445–456 to characterize the C-termini of KP28 and KP28-L (Fukuda et al., 1988). Both proteins contained the carboxyl-terminal epitopes. On the basis of these results, we concluded that KP28-L contained an extra ~30 amino acids at the carboxyl terminus which are unrelated to h-TRβ1. It is unclear to us why an additional 30 amino acids were synthesized in *E. coli*.

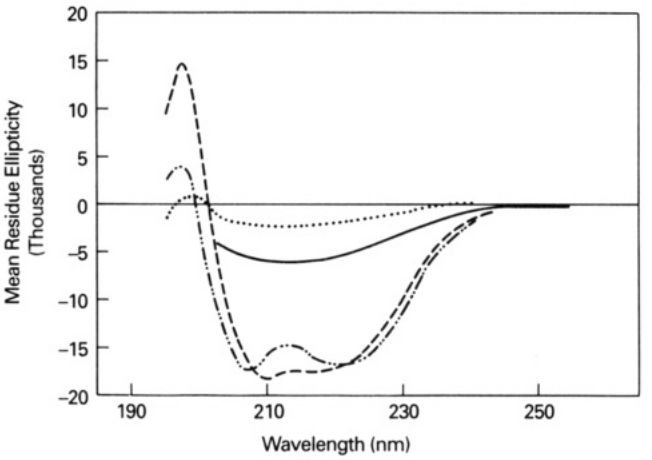

FIGURE 3: Ultraviolet circular dichroism spectra of fragments of h-TRβ1 truncated from the amino terminus: (–•–) MD32; (– –) KD29; (—) DD28; (•••) KD25. Spectra were recorded at 25 °C on a Jasco J-500C spectorpolarimeter, using a DP-500N data processor and 1 mm pathlength quartz cuvettes, in 0.1 M sodium phosphate buffer, pH 7.2. Spectra were digitized and downloaded as before (McPhie & Shrager, 1992) and analyzed in terms of secondary structure by a least-squares fit using the PC-MLAB computer program (Civilized Software, Inc., Bethesda, MD). Protein concentrations (50–100 μg/mL) were measured by amino acid analysis.

The purified proteins were characterized by T_3 binding. The K_a values for MD32 and KD29 were 7.9×10^8 and 4.8×10^8 M^{-1}, respectively. The K_a's are virtually identical to those reported previously (Lin et al., 1991). DD28, KD25, and KP28 did not bind T_3. These results are similar to those described earlier for the highly enriched proteins (Lin et al., 1991).

Secondary Structure of HBD. Figure 3 shows the far-ultraviolet CD spectra of fragments MD32, KD29, DD28, and KD25. MD32 and KD29 both have intense spectra, with minima at 222 and 208–210 nm and maxima around 190–195 nm, characteristic of α-helical structures. The spectra of DD28 and KD25 were greatly reduced in intensity, with a single minimum around 215 nm, a wavelength usually assigned to β-sheet structures. None of these spectra were altered by the addition of up to 2-fold molar excesses of L-T_3 or of 3,3′,5-triiodothyroacetic acid.

It was more difficult to obtain data for KP28 due to the copurification of KP28-L. However, this problem was overcome by measuring the spectra of column fractions containing varying amounts of these two proteins. The relative concentrations of the two proteins were estimated from gels, and their intrinsic CD spectra (shown in Figure 4) were calculated by computer analysis.

Effect of Temperature on Structure. Single wavelength melting curves, following ellipticity at 215 nm (Figure 5), showed that increasing temperature produced cooperative sigmoidal thermal transitions in both MD32 and KD29 with extensive loss of secondary structure (T_m = 40 °C, in each case), whereas the ellipticities of DD28 and KD25 both varied linearly with temperature. After overnight cooling of these solutions, only 50% of the original ellipticities were recovered, indicating that the thermal unfolding must be irreversible.

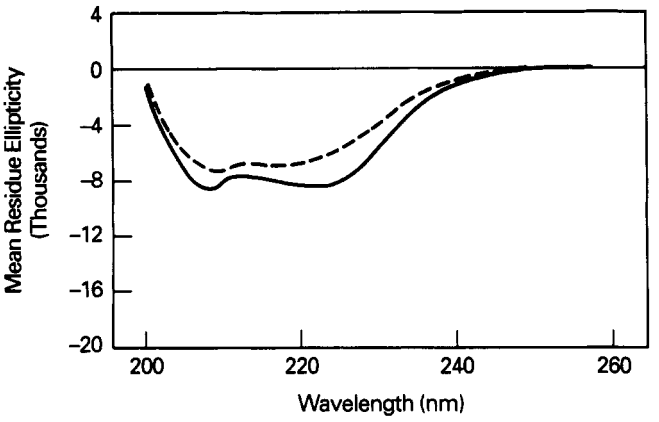

FIGURE 4: Resolved CD spectra of KP28. CD spectra were measured for three column fractions containing varying proportions of KP28 and KP28-L, as described in Figure 3. The relative amounts of the two proteins in each fraction were estimated densitometrically after their sepration by SDS–PAGE. The experimental spectra were then resolved into the intrinsic CD spectra of KP28 (—) and KP28-L (- - -) by matrix analysis, using PC-MLAB.

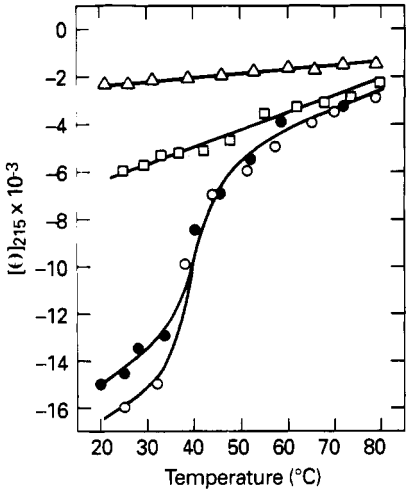

FIGURE 5: Thermal unfolding curves of truncated h-TRβ1 measured by CD changes at 215 nm: (●) MD32; (○) KD29; (□ DD28; (△) KD25. Curves were recorded on a Jasco J-500C spectropolarimeter as before (McPhie & Shrager, 1992). Protein solutions (50–100 μg/mL) in 0.1 M sodium phosphate buffer, pH 7.2, were in 1 mm pathlength quartz cuvettes held in a brass cell holder and thermostated by a Lauda circulating water bath. Temperatures in the cuvettes were measured with a Yellow Springs Instruments thermocouple.

Consequently, no thermodynamic analyses of these transitions were attempted.

Effect of pH on Structure. Binding of thyroid hormone to intact h-TRβ1 can be detected over the pH range 5.5–9.0 (Lin et al., 1989). The secondary structure of fragment MD32 was found to be independent of pH over this range (data not shown). However, deletion of residues 169–201 destabilizes the HBD against variations in pH. Fragment KD29 was found to be partially unfolded at the extremes of this pH range (Figure 6). Analysis of these spectra, as described in Table I, indicated that fragment KD29 contained around 44% α-helix and no β-strands at pH 5.5 and 50% α-helix and 8% β-strands at pH 9.0. At pH 7.0, in the presence of 6 M guanidine hydrochloride, both fragments showed CD spectra characteristic of randomly coiled polypeptide chains (Figure 6).

Analysis of Secondary Structure. Far-ultraviolet CD spectra are thought to reflect the conformation of polypeptide chains in solution. Problems in the quantitative analysis of such spectra in terms of reference spectra of standard secondary

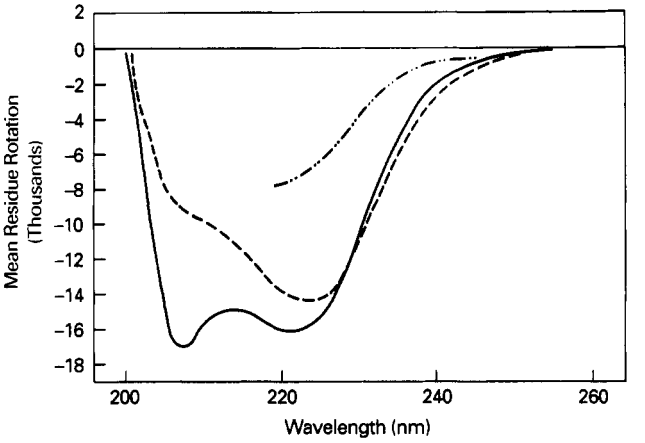

FIGURE 6: Effect of pH on CD spectra of KD29. Spectra were measured at 25 °C for three solutions of KD29 in 0.1 M sodium phosphate adjusted to pH 5.5 (- -), pH 9.0 (—), and pH 7.2 (-•-) plus 6 M guanidine hydrochloride.

Table I: Fractional Secondary Structure Resolved from CD Spectra

protein	α-helix	β-sheet	remainder
MD32	0.43 ± 0.04	0.16 ± 0.13	0.41 ± 0.19[a]
	0.58 ± 0.01	0.05 ± 0.04	0.38 ± 0.05[b]
KD29	0.51 ± 0.05	0.27 ± 0.15	0.22 ± 0.22[a]
	0.57 ± 0.01	0.24 ± 0.06	0.19 ± 0.07[b]
DD28	0.20 ± 0.02	0.22 ± 0.07	0.58 ± 0.09[a]
	0.17 ± 0.02	0.20 ± 0.07	0.63 ± 0.09[b]
KD25	0.11 ± 0.04	0.24 ± 0.13	0.64 ± 0.19[a]
	0.05 ± 0.02	0.18 ± 0.09	0.77 ± 0.11[b]
KP28	0.25 ● 0.02	0.06 ± 0.05	0.69 ± 0.07[a]
	0.29 ± 0.01	0.05 ± 0.04	0.66 ± 0.07[b]

[a] Using base spectra of Chang et al. (1976). [b] Using base spectra of White (1976).

structures and appropriate wavelength ranges have been discussed recently by Venyaminov et al. (1991). We analyzed our spectra using the reference spectra of Chang et al. (1976) and White (1976), which are derived from different sets of reference proteins and wavelength ranges. The results of these analyses, shown in Table I, show reasonably good agreement in the estimated amounts of α-helix, β-sheet, and "remainder" in the various fragments. More importantly, without regard to the precise quantitative aspects of these analyses, the experimental data show drastic changes in the structure of fragments DD28, KD25, and KP28 resulting from truncation of their sequences, compared to MD32 and KD29.

DISCUSSION

On the basis of genetic experiments and DNA sequence homologies, proteins in the steroid/thyroid hormone receptor superfamily have been assigned functional domains, A–E (Evans, 1988). Domain C has been shown to contain two zinc finger structures (Luisi et al., 1991), one of which binds to DNA and the second is involved in the dimerization of DNA-bound receptor molecules. Domain D links these to the postulated hormone binding domain E and is thought to carry a nuclear localization sequence (Dingwall & Laskey, 1991). Nothing is known of the structure of the rest of the molecule or the basis of the interactions between these domains, but information may be obtained by physical studies on the protein and its separate domains, which allows us to propose a speculative model of its structure.

Figure 3 and Table I indicate that fragment MD32 (all of domains D and E) contains extensive regions of α-helix and β-sheet. The thermal unfolding curve (Figure 5) shows the

highly cooperative nature of this structure, typical of a native globular protein. The CD spectrum and melting curve of KD29 are distinct from those of MD32, but are still consistent with the presence of large amounts of cooperative secondary structure in the protein. However, deletion of the next 10 amino acids to produce DD28 results in drastic changes in the CD spectrum, reflected largely as a loss of α-helix in Table I. Continued truncation of the sequence to yield KD25 (all of domain E) produces larger changes in the CD spectrum, indicating further loss of secondary structure. Neither DD28 nor KD25 showed sigmoidal thermal unfolding curves, consistent with a lack of cooperative structural interactions in these proteins. Likewise, the results in Figure 4 indicate that the structure of fragment KD29 is extensively disrupted by the removal of eight carboxyl-terminal amino acids to give KP28 and is not restored by the addition of an unrelated carboxyl-terminal sequence (KP28-L). Loss of cooperative secondary structure in these protein fragments correlates with their loss of ability to bind thyroid hormone (Lin et al., 1991). Shortening KD29 to produce these fragments results in much longer losses of secondary structure than could be contained in the deleted region (Table I, Figure 1). Thus, these regions must be essential for the structural integrity of the hormone binding domain.

Selection of these fragments of h-TRβ1 for use in our earlier studies on hormone binding was based on a prediction of the receptor's secondary structure according to Garnier et al. (1978). More recently, Niermann and Kirschner (1990) have shown that improved accuracy results by combining different prediction methods with considerations of side-chain hydrophobicity and backbone flexibility and by averaging the calculations over the sequences of homologous proteins believed to have the same fold. To help in the interpretation of our CD results, we have used their methods on the seven thyroid hormone receptor sequences and alignments shown in Yaoita et al. (1990), together with the mouse (Masuda et al., 1990) and human sequences (Sakurai et al., 1990). The conclusions of this new analysis, reported in Figure 1B, show minor but significant differences from our previous results (Lin et al., 1991). The secondary structural elements predicted to be present in domains D and E of h-TRβ1 are compatible with the observed CD spectrum of MD32, consisting of extensive, alternating stretches of α-helix and β-strand. Such arrangements are found in two main types of tertiary fold: an α/β barrel or a curved β-sheet flanked by α-helices (Hurle et al., 1987).

Our results can most readily be explained in terms of the structures of α/β barrel proteins (Farber & Pestko, 1990). Some α/β barrels are preceded by an extra α-helix, which blocks the N-terminus of the barrel. Deletion of predicted helix 0 has little effect on the barrel, reflected by the retention of secondary structure and hormone binding affinity in fragment KD29. Helix 0 may serve to link this region to DNA binding domain C, which can modulate HBD's hormone affinity (Lin et al., 1991). Deletion of the next 10 amino acids (predicted to contain the first strand of the β-barrel), an essential structural element, produces a drastic reduction in the secondary structure of the protein and abolishes hormone binding activity. Further loss of structure results from removal of the next 24 residues, which are predicted to contain the α/β barrel's first helix. Extensive unfolding also results from the removal of eight residues from the carboxyl terminus of the protein. Our model predicts that this forms the last helix of the α/β barrel, which is another essential region, and places it next to amino-terminal strand 1.

Some α/β barrel proteins contain extra helices between their main structural elements (Farber & Pestko, 1990). Hydrophobicity patterns suggest that helix 4 packs against the β-sheet-forming part of the α/β barrel and that helix 4′ is an extra helix on the barrel's N-terminal face. The conformation of this region of the molecule is particularly important, since many of the natural 15 point mutations identified in the β1 gene from the affected kindred of generalized thyroid hormone resistance are clustered in this region (Refetoff, 1992; Behr & Loos, 1992; Meier et al., 1992) (see Figure 1B). Our model places two of these mutations in helix 4 (M^{305} and A^{312}) and two in strand 8 (P^{448} and L^{449}), where they may affect the general stability and conformation of this domain. The other sites (A^{229}, R^{315}, G^{327}, T^{332}, Q^{335}, G^{340}, R^{433}, M^{437}, T^{443}, and F^{446}) are all predicted to fall on the N-terminal face of the barrel close together and capable of interaction with the DNA binding domain, which is known to modulate the affinity of the receptor for hormone (Lin et al., 1992).

Extensive homologies in amino acid sequence have been detected among the hormone binding domains of members of this superfamily of receptors (Evans, 1988), suggesting an underlying similarity in their structures. Recently, Toney et al. (1993) published CD spectra of intact chicken α1 thyroid hormone receptor and analyzed their data to show extensive regions of α-helix and β-sheet. Their results are compatible with an α/β barrel structure for the HBD. For enzymes which share the α/β barrel structure, substrate binding specificity is dictated by residues in loops (Farber & Pestko, 1990). The same may be true for this family of receptors. Other studies have indicated that the region between residues 281 and 300 is involved in receptor dimerization (Forman et al., 1989) and binding to other nuclear proteins (O'Donnel et al., 1991). Deleterious point mutations were demonstrated at residues A^{282}, K^{283}, L^{285}, and D^{295}. A^{282} and L^{285} are conversed hydrophobic residues, which in our model link helix 3 to the β-barrel. Replacement with hydrophilic residues could destabilize this region of the protein. K^{283} and D^{295} are strongly conserved, exposed, charged residues which may provide specificity for the association reactions. The trypsin-sensitive site, previously detected in a h-TRβ1/T_4 complex, at K^{239}–F^{240} (Lin et al., 1991) is predicted to fall in a large loop on the surface between helix 1 and strand 2.

Thyroid hormone binding sites have been identified as a β-barrel in transthyretin (Oately et al., 1984) and modeled as a β-barrel in thyroxine binding globulin (Terry & Blake, 1992). Our recent studies on p58, a ubiquitous thyroid hormone binding protein, showed it to be an isozyme of pyruvate kinase (Kato et al., 1989), whose structure includes both an α/β barrel and extensive regions of β-sheet (Muirhead et al., 1986). These findings are consistent with our proposed α/β barrel model for the HBD of h-TRβ1 and suggest that the hormone binds from the proposed barrel's carboxyl-end face. Our results suggest a simplified structure of h-TRβ1 consisting of two functional domains: the DNA binding domain of known structure (residues 102–169), linked by an α-helix to a hormone binding domain (residues 201–456) with an α/β barrel structure. While highly speculative and awaiting verification by crystallographic analysis, this model provides a basis for future investigations using protein chemistry and site-specific mutagenesis.

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