Analysis of the Binding of 3,3′,5-Triiodo-L-thyronine and Its Analogues to Mutant Human β1 Thyroid Hormone Receptors: A Model of the Hormone Binding Site

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ABSTRACT: To understand the nature of the thyroid hormone binding site, we characterized the binding of 3,3′,5-triiodo-L-thyronine (T_3) and its analogues to eight naturally occurring mutated human β1 thyroid hormone receptors (h-TRβ1). The mutant receptors were derived from patients with the syndrome of generalized thyroid hormone resistance, and each has a point mutation in the hormone binding domain (KT, R338W; TP, L450H; IR, D322H; NN, G347E; AH, P453H; OK, M442V; RL, F459C; and ED, A317T). Compared to the wild-type h-TRβ1, binding of T_3 was reduced by as much as 97% for the mutants. The order of binding affinity of wild-type h-TRβ1 to the analogues is T_3 > D-T_3 > L-thyroxine > 3,5-diiodo-L-thyronine > 3,3′,5′-triiodo-L-thyronine. The mutant receptors showed essentially the same order of reduced affinities for the analogues, but the amounts of the reductions varied in each case. These results suggest specific local interactions (interplay) of analogues with the mutated residues in the receptors. On the basis of these data and a putative structure of the hormone binding domain as an eight-stranded α/β barrel, we propose the location of the hormone in the binding site of h-TRβ1. Ionic bonds anchor the hormone's alanine side chain to loop 4 of the 8-fold α/β barrel. The phenyl ring lies across the amino-terminal face of the domain with the phenoxy ring pointing downward into the barrel interacting with β-strand 8 on the opposite side. Loops 1 and 7, which are located on the same face as the DNA binding domain, fold over the top of the barrel toward the bound hormone. The T_3-induced conformational changes observed by us and others may be partly mediated by loops 1 and 7. The present model should provide a basis for further studies to understand the T_3-dependent transcriptional activity of h-TRβ1.

Thyroid hormone nuclear receptors (TRs)[1] are members of the steroid hormone/retinoic acid receptor superfamily. They are ligand-dependent transcriptional factors which regulate growth, differentiation, and development. Two major TR isoforms have been isolated, TRα and TRβ, whose genes are located on chromosomes 17 and 3, respectively (Chin, 1991). By characterization of the promoter regions of T_3 response genes, the consensus sequence of T_3 response elements (TREs) has been identified to be AGGT(C/G/A)A. These half-site binding motifs could be oriented as palindrome, direct repeat separated by proper gaps or inverted palindrome (Chin, 1991). The interaction of TRs with TREs is further modulated by other nuclear proteins including various forms of the retinoid receptor (Zhang, 1992; Marks *et al.*, 1992; Hallenbeck, 1992).

The transcriptional activity of TRs is T_3-dependent. At the present time, the molecular basis for the T_3 dependence is not clearly understood. It has been shown that binding of T_3 induces the dissociation of TR homodimer bound to DNA, thereby relieving the suppression effects of the unliganded TR (Yen *et al.*, 1992; Meier *et al.*, 1993). Recently, using an *in vitro* transcriptional assay, Findell *et al.* have demonstrated that the unliganded TR is an active transcriptional repressor. The repression by TR is relieved when TR is bound to ligand due to a possible conformational change that disrupts the repressor function (Findell *et al.*, 1993). We have recently demonstrated directly that T_3 indeed induces conformational changes of human β1 TR (h-TRβ1). We found that binding of T_3 to h-TRβ1 altered the susceptibility of several sites to tryptic and chymotryptic cleavage (Bhat *et al.*, 1993).

The conformational changes could be due to a global effect resulting from a more compact hormone binding domain induced by T_3 binding. It could also be derived from an alteration of the local structures which are in direct contact with T_3. To further delineate the molecular basis of the hormone-induced structural changes in TR, the present studies analyzed the interaction of T_3 and its analogues with wild-type and mutated h-TRβ1. We utilized the naturally occurring mutant receptors which were derived from thyroid hormone resistant patients (Mixson *et al.*, 1992; Parrilla *et al.*, 1991; Usala *et al.*, 1990). These mutant receptors have point mutations in the hormone binding domain which result in the reduction of T_3 binding affinity (Mixson *et al.*, 1992; Usala *et al.*, 1990; Meier *et al.*, 1992). This provides us with a means to probe the nature of the T_3 binding site in h-TRβ1. Because of the lack of definitive data (X-ray crystallography

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[1] Abbreviations: T_3, 3,3′,5-triiodo-L-thyronine; T_4, 3,3′,5,5′-tetraiodo-L-thyronine; T_2, 3,5-diiodo-L-thyronine; r-T_3, 3,3′,5′-triiodo-L-thyronine; TR, thyroid hormone nuclear receptor; h-TRβ1, human β1 TR; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

or NMR) on the structures of this receptor family, we recently suggested a putative structure of the hormone binding domain of h-TRβ1 as an 8-fold α/β barrel (McPhie *et al.*, 1993). By characterization of the binding of T_3 and its analogues to eight mutants, the present studies extend this model of the hormone binding site in 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 obtained from Research Organics, Inc. (Cleveland, OH). L-T_3, D-T_3, L-T_4, r-T_3, and phenylmethanesulfonyl fluoride were purchased from Sigma (St. Louis, MO). Aprotinin and leupeptin were obtained from Boehringer Mannheim (Indianapolis, IN).

Construction of Expression Plasmids. The mutants studied are listed in Table 1. The characterization and identification of the mutation sites have been described (Usala *et al.*, 1990a; Parrilla *et al.*, 1991; Mixson *et al.*, 1992). The mutation sites of ED, IR, KT, and NN are located in exon 9 of the h-TRβ gene and those of OK, TP, AH, and RL in exon 10. Different strategies were used to construct the T7 expression plasmids for these two groups of mutants. However, all mutants were cloned into the same parental T7 expression plasmid pRCJ. This plasmid was derived from pCJ3 (Park *et al.*, 1993) by deletion of a *Bgl*II site in the noncoding region of the plasmid and inserting a *Hin*dIII site at 3′ of the poly(A) site.

For the exon 10 mutants, the DNA fragments containing the mutation site were cloned into *Bgl*II and *Bam*HI sites of pRCJ similarly as described by Mixson *et al.* (1992). For the exon 9 mutants, the plasmids were constructed by using the splicing overlap extension method described in Mixson *et al.* (1993). The sequences in the mutants were confirmed by sequencing.

Expression of the Mutant h-TRβ1 Proteins. The expression of the mutant h-TRβ1 proteins in BL21/LysS cells was under the control of the T7 promoter (Studier *et al.*, 1990). BL21/LysS cells containing the plasmid were grown in LB broth with 100 μg/mL ampicillin and 15 μg/mL chloramphenicol to an absorption at 600 nm of 0.45–0.55. The expression of proteins was induced with 1 mM isopropyl β-D-thiogalactopyranoside for 2 h at 37 °C. After induction, the cells were chilled on ice and centrifuged for 15 min at 2000*g*. The supernatant was discarded. The pellet was frozen in dry ice and further processed as described below or kept at −70 °C until ready to be used.

The frozen cell pellet from above was thawed for 10 min at 22 °C. Two milliliters of the lysis buffer containing 25 mM Tris·HCl, pH 8.0, 1 mM dithiothreitol, 50 mM KCl, 0.5 M NaCl, 0.01% Lubrol, 10% glycerol, 0.5 mM phenylmethanesulfonyl fluoride, 20 μg/mL aprotinin, and 1 μg/mL leupeptin was added to the pellet derived from 10 mL of cell culture. The cell pellet was suspended and lyzed by vigorous pipeting. An additional 18 mL of lysis buffer in which 0.5 M NaCl was omitted was added, and the suspension was centrifuged for 15 min at 27000*g*. The expression of the h-TRβ1 mutant protein in the cellular extracts was analyzed by 10% SDS–PAGE, Western blotting, and T_3 binding studies.

Western Blotting. Western blotting was carried out similarly as described by Obata *et al.* (1987). Briefly, the expressed wild-type and mutants (~0.5 μL) obtained above were analyzed by 10% SDS–PAGE. After gel electrophoresis, the proteins were blotted onto nitrocellulose paper (0.45 μm). The paper was blocked with normal goat serum followed by reacting with anti-h-TRβ1 antibodies N-98 (1:1000 dilution) or C-91 (1:3000 dilution). After the primary antibodies were washed off, the blots were treated with Vectastain ABC reagents (Vector Lab, CA), and the color was developed using 3,3′-diaminobenzidine.

Binding of T_3 and Its Analogues to h-TRβ1 Mutants. The lysate prepared from above was incubated with 0.5 nM [^{125}I]-T_3 for 20 h at 4 °C in the absence or presence of increasing concentrations of unlabeled L-T_3, D-T_3, T_4, and r-T_3. The receptor-bound T_3 was separated from unbound T_3 by the filter binding assay and by the gel filtration assay described (Usala *et al.*, 1990; Lin *et al.*, 1991).

The binding constants (K_a) were calculated using the computer program LIGAND (Munson & Rodbard, 1980). Free energies of binding of analogues (L) to the wild-type (WT) and mutant receptors (M) were calculated using the relationship:

$$\Delta G = -RT \ln K_a \quad \text{(i)}$$

$\Delta\Delta G_{L,M}$ values, changes in the average free energies of binding of analogues to the wild-type and mutant receptors ($\Delta G_{L,M}$) relative to the free energy of L-T_3 binding to wild-type h-TRβ1 (ΔG_{WT}), were calculated as

$$\Delta\Delta G_{L,M} = \Delta G_{L,M} - \Delta G_{WT} \quad \text{(ii)}$$

RESULTS

Expression of the Mutant Receptors in Escherichia coli. The expression of the wild-type and mutant h-TRβ1 in *E. coli* is under the control of the T7 promoter. Upon induction by isopropyl β-D-thiogalactopyranoside, the receptor proteins were expressed (Studier *et al.*, 1990). In contrast to the cells which were not transformed by a T7 expression vector (lane 11 of Figure 1A), a major protein with the expected apparent molecular weight of 55 000 was detected for all mutants listed in Table 1. For comparison, lane 1 of Figure 1A shows the highly enriched wild-type h-TRβ1 obtained according to Lin *et al.* (1990). Lanes 2–10 indicate that the mutant receptors expressed similarly as the wild-type receptor (lane 2 vs lanes 3–10).

The coding sequences of the mutant receptors were all verified by nucleotide sequencing. However, to be certain that no minor proteolytic cleavage occurred after expression, we carried out Western blotting using two anti-peptide antibodies. The epitopes of N-98 and C-91 are at the amino terminus (Cys^{20}–Glu^{34})[2] and the carboxyl terminus (Cys^{446}–Asp^{461}), respectively (Fukuda et al., 1988). As shown in Figure 1B and Figure 1C, all mutant receptor proteins are recognized by both antibodies. These results indicate that the expressed mutant receptors contain both the amino and carboxyl termini; therefore, the expressed receptors are intact.

Binding of T_3 and Analogues to the Wild-Type and Mutant Receptors. The binding of L-T_3 to wild-type h-TRβ1 and to mutants, ED, IR, KT, NN, OK, TP, AH, and RL, was measured. In all cases, the point mutations in these proteins lead to reductions in their binding affinities, compared to the wild-type receptor (Mixson *et al.*, 1992; Usala *et al.*, 1990; Meier *et al.*, 1992). ED is the most deleterious mutant with

[2] The numbering system used for the amino acid sequence and point mutation sites of h-TRβ1 differs from that used in our previous publications. Following the recommendations of the First Workshop on Thyroid Hormone Resistance, all sequence numbers are increased by five, reflecting the presence of another five amino acids at the N-terminus of the deduced amino acid sequence (V. K. K. Chatterjee, personal communication).

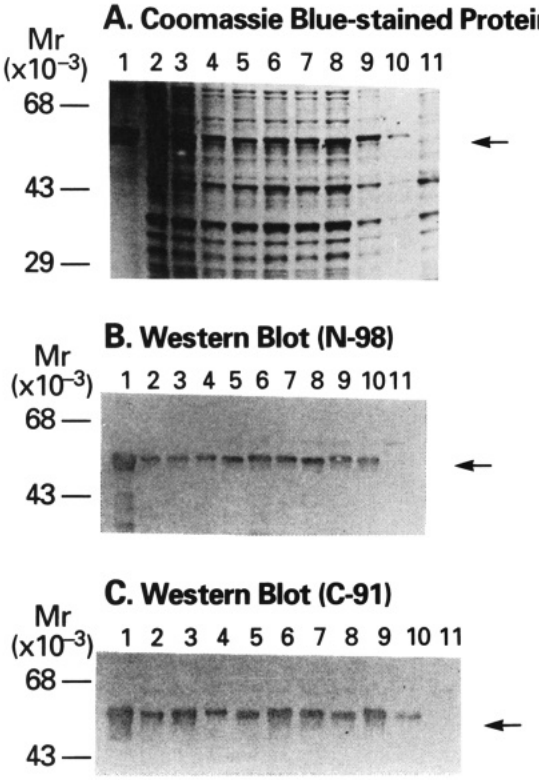

FIGURE 1: Expression of the wild-type and mutant h-TRβ1 in *Escherichia coli*. After expression of the wild-type and mutant h-TRβ1 as described under Materials and Methods, 1.5 mL of cell suspension was centrifuged, and the cells were lysed by boiling in 100 μL of sample buffer. (A) Five microliters of lysate was loaded onto each lane. After SDS–PAGE, the gel was stained by Coomassie Blue. (B) and (C) are Western blots using antibodies N-98 and C-91, respectively; 0.5 μL of the lysates was loaded onto each lane. After SDS–PAGE, the proteins were transferred onto nitrocellulose paper and reacted with N-98 or C-91 according to Fukuda *et al.* (1988). The order of the proteins in lanes 1–11 is highly purified wild-type h-TRβ1 (5 μg in panel A and 0.5 μg in panels B and C, respectively) and lysate of the wild-type h-TRβ1, ED, IR, KT, NN, OK, TP, AH, RL, and nontransformed BL21/LysS cells. The arrows indicate the expressed wild-type h-TRβ1 or mutant h-TRβ1.

Table 1: h-TRβ1 Mutants

kindred	exon	mutation	location in predicted structure of hormone binding domain[a]
ED	9	A317T	helix 4
IR	9	D322H	loop 4
KT	9	R338W	loop 4
NN	9	G347E	β-strand 5
OK	10	M442V	loop 7
TP	10	L450H	β-strand 8
AH	10	P453H	β-strand 8
RL	10	F459C	helix 8

[a] See McPhie *et al.* (1993).

only 3% of the wild-type receptor's affinity for L-T_3. For a better understanding of the molecular basis of these reductions, we further characterized the binding site by using T_3 analogues as probes. The analogues used were D-T_3, L-thyroxine (T_4), 3,5-diiodo-L-thyronine (T_2), and 3,3′,5′-triiodo-L-thyronine (r-T_3) (see Figure 2). Typical competitive binding curves for the wild-type h-TRβ1 and four representative mutants are shown in Figure 3. All of the analogues had lower affinities than L-T_3 for the wild-type receptor (L-T_3 > D-T_3 > T_4 > T_2 > r-T_3). Mean values of binding constants (K_a) for each combination of hormone analogue and receptor, derived by analysis of binding data as measured by the filter binding assay, are shown in Table 2. Identical results were also

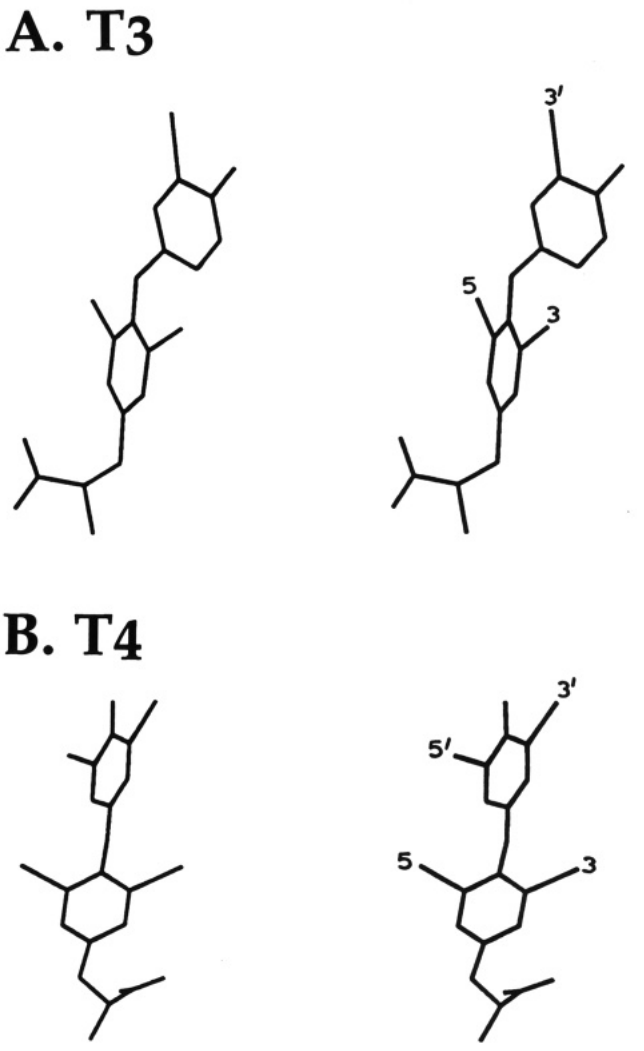

FIGURE 2: Stereoview showing the structure of (A) L-T_3 (Cody & Duax, 1973) and (B) L-T_4 (Cody, 1981). Hydrogen atoms are not shown in these figures. The hormones appear in the ionized forms. Other analogues studied are 3,3′,5-triiodo-D-thyronine (D-T_3), 3,5-diiodo-L-thyronine (T_2), and 3,3′,5′-triiodo-L-thyronine (r-T_3). The molecules are approximately 15 Å long.

obtained using the gel filtration assay. Free energies of binding were calculated using eq i and are listed in Table 3.

From the K_as (Table 2) and ΔGs (Table 3), it is clear that the mutant receptors showed essentially the same order of reduced affinities for the analogues, but the amounts of the reductions varied in each case, reflecting specificity in the ligand–protein interactions. This derives from the many specific local interactions which have been shown to play an important role in determining binding specificity in enzymatic catalysis and ligand binding to proteins (Bone & Aggard, 1991; Wells, 1990). To evaluate these local effects, we calculated values of $\Delta\Delta G_{L,M}$ which measure the changes in the average free energies in the binding of analogues to the wild-type and mutant proteins ($\Delta G_{L,M}$) relative to the free energy of L-T_3 binding to wild-type h-TRβ1 (ΔG_{WT}). The $\Delta\Delta G_{L,M}$ values were calculated according to eq ii under Materials and Methods and are listed in Table 4. We assumed initially that these values are additive functions of free energy changes resulting from separate alterations in the structure of the ligand (Dietrich *et al.*, 1977) and of the protein (Bone & Agard, 1991). Thus, each experimental value should be the sum of the change in free energy from the structural change in the ligand binding to wild-type protein ($\Delta\Delta G_L$, Table 4, column 2) plus the change in free energy of binding of L-T_3,

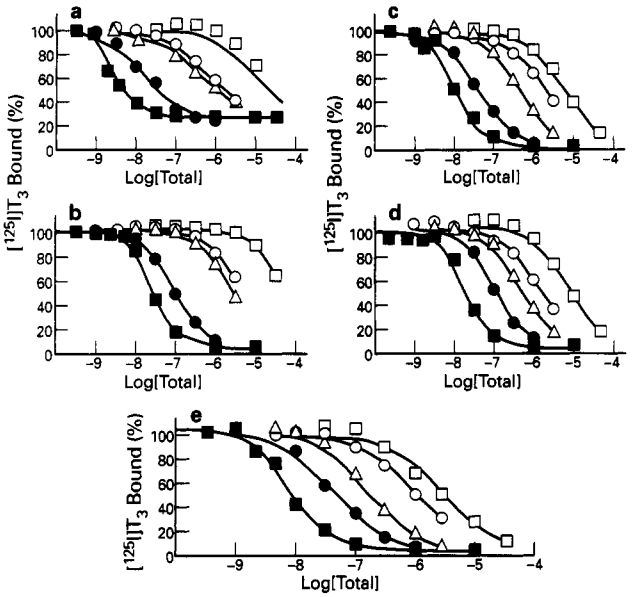

FIGURE 3: Binding of T_3 and its analogues to the wild-type and mutant h-TRβ1. The lysate containing the wild-type or mutant receptors was incubated with 0.5 nM [^{125}I]T_3 in the absence or presence of increasing concentrations of unlabeled L-T_3 (■), D-T_3 (●), L-T_4 (△), T_2 (○), or r-T_3 (□) at 4 °C for 20 h. The free and bound [^{125}I]T_3 were separated by the filter binding assay as described under Materials and Methods. (a) Wild-type h-TRβ1; (b) ED; (c) NN; (d) OK; and (e) RL.

resulting from the amino acid mutation in the receptor ($\Delta\Delta G_M$, Table 4, row 3):

$$\Delta\Delta G_{L,M} = \Delta\Delta G_L + \Delta\Delta G_M \quad \text{(iii)}$$

Experimental values of free energy changes are plotted against predicted, additive values in Figure 4. If our assmption of additivity of free energies is correct, the data points should fall on the diagonal of this plot. Within experimental error, this is true for most combinations of ligand and mutant protein. Those combinations whose experimental values show significant deviations from their predicted values [$>RT = 0.6$ kcal/mol] are underlined in Table 4 and marked in Figure 4. The distribution of the deviations of the remaining combinations of protein and ligand from predicted behavior had a standard error of ±0.2 kcal/mol. Thus, those combinations judged "significant" were at least three standard errors away from the expected behavior.

Following the example of proteins with double mutations (Wells, 1990), we take such nonadditivity to show direct or indirect interactions between the mutated amino acid and the "mutation" in the ligand. We will refer to this phenomenon as "interplay", to distinguish it from specific ligand–receptor interations, as normally understood. In all cases, this interplay serves to partially alleviate the deleterious effects of the two changes in the receptor and the ligand. No examples were found with a reduction of the free energy of binding significantly larger than predicted, which would indicate that the structural changes reinforced each other. Thus, these mutations reduce the affinity of the protein for all analogues and lessen the specificity of the receptor for its optimal ligand, L-T_3.

DISCUSSION

The present studies evaluated the binding of five T_3 analogues to eight mutant receptors. The free energies of binding of these analogues to the wild-type h-TRβ1 are in good agreement with those determined previously by using the TRs in intact rat liver nuclei and nuclear extracts (Dietrich *et al.*, 1977). Our data indicate that the T_3 binding site in the recombinant h-TRβ1 is indistinguishable from that in the rat liver nuclei (Dietrich *et al.*, 1977). Dietrich *et al.* analyzed their more extensive data by quantitative structure–activity correlations and showed that experimental free energies of binding could be partitioned into additive, hydrophobic, and steric contributions from individual substituent groups on the diphenyl ether thyronine nucleus (see Figure 2). Their conclusions on the mechanism of binding of analogues to receptor are summarized as follows:

(1) Binding is enhanced by bulky, lipophilic 3 and 5 substituents in the phenyl ring. These serve to orient the planes of the two aromatic rings perpendicularly with the 3′-iodine distal to the phenyl ring.

(2) Binding is also enhanced by 3′ lipophilic substituents of limited size and decreased by any 5′ substituents in the phenoxy ring.

(3) Binding requires an unionized 4′-OH, which probably donates a hydrogen bond to the 5′ side of the nuclear receptor binding site. They postulated a model in which the hormone's carboxylate side chain forms an ionic bond with a group on the receptor. The phenyl ring of the hormone lies flat on a hydrophobic surface with the phenoxy ring inserted into a narrow well in the receptor. From our present results, we may add the observation that the chiral conformation of the alanine side chain is relatively unimportant as a determinant of binding (Table 2).

Thyroid hormone binding sites have been determined in transthyretin (De la Paz *et al.*, 1992) and modeled in thyroxine binding globulin (Terry & Blake, 1992). These two plasma proteins have been divided into three regions: (A) a charged region with side chains which form ion pairs with the zwitterionic alanine side chain of the ligand; (B) a hydrophobic patch formed from the hydrocarbon side chains of β-sheet residues which bind the aromatic rings and the iodine atoms; (C) a hydrophilic patch containing serines and threonines which accommodates the phenolic hydroxyl group.

There may be differences in the structure of these hormone binding sites from that of the nuclear receptor, but it is difficult to imagine a drastically different arrangement which will accommodate this family of ligands with such high affinity. h-TRβ1 shows different specificities for ligands (e.g., $T_3 > T_4 > T_2$) than these proteins ($T_4 > T_3 > T_2$). Dietrich *et al.* (1977) ascribed this to variations in phenolic pK values in the series of analogues, implying that the nuclear receptor binds hormones with the phenolic group unionized, whereas the transport proteins bind the phenoxide ion. Thus, one may anticipate an absence or decrease in size of the hydrophilic patch in the binding site of h-TRβ1. Otherwise, this scheme corresponds well with the hypothetical model for the nuclear hormone binding site of Dietrich *et al.* (1977).

Our current knowledge of protein–ligand binding indicates that intermolecular interactions occur over large surface areas involving contacts between numerous regions of the ligand and many amino acid residues in the protein (Bone & Agard, 1991). These interactions can be modulated by variation in the structure of the ligand or by mutagenesis of the protein. We have combined these two approaches in a study of the binding site of h-TRβ1 by measuring the free energies of binding of a series of thyroid hormone analogues (see Figure 2) to wild-type h-TRβ1 and to eight single point mutant forms of the receptors cloned from patients with generalized

Table 2: Affinity Constants of T_3 Analogues to Wild-Type and Mutant Receptors[a]

	$K_a \times 10^8$ (M^{-1})								
analogues	WT	KT	TP	IR	AH	NN	RL	OK	ED
T_3	150	52	9.3	7.3	5.7	5.9	5.5	5.4	3.8
D-T_3	15	3.5	1.4	1.3	0.88	0.88	0.65	0.63	0.65
T_4	0.38	0.11	0.10	0.047	0.066	0.096	0.18	0.13	0.02
T_2	0.24	0.10	0.02	0.011	0.014	0.018	0.027	0.034	0.011
r-T_3	0.026	0.0056	0.0068	0.0022	0.004	0.0075	0.009	0.0089	0.00086

[a] Affinity constants were calculated by analysis of data shown in Figure 3 using the computer program LIGAND (Munson & Rodbard, 1980). Standard errors of the derived values lay in the range 3–10% within the same experiments.

Table 3: Free Energies of Binding of Thyroid Hormone Analogues to Wild-Type and Mutant Receptors

	ΔG (kcal/mol)[a]								
analogues	WT	KT	TP	IR	AH	NN	RL	OK	ED
T_3	−12.99	−12.39	−11.44	−11.30	−11.17	−11.19	−11.15	−11.13	−10.94
D-T_3	−11.70	−10.90	−10.37	−10.33	−10.14	−10.13	−9.97	−9.95	−9.97
T_4	−9.67	−8.97	−8.93	−8.51	−8.70	−8.91	−9.26	−9.08	−8.03
T_2	−9.41	−8.95	−8.04	−7.72	−7.82	−7.96	−8.20	−8.33	−7.71
r-T_3	−8.18	−7.33	−7.44	−6.81	−7.15	−7.50	−7.59	−7.59	−6.29

[a] Free energies of binding were calculated using eq i from binding constants shown in Table 2.

Table 4: Changes in Free Energies of Binding to Mutant Receptors Versus That of L-T_3 Binding to the Wild-Type h-TRβ1

	$\Delta\Delta G$ (kcal/mol)[a]								
analogues	WT	KT	TP	IR	AH	NN	RL	OK	ED
T_3	0.00	0.59	1.55	1.69	1.82	1.80	1.84	1.85	2.05
D-T_3	1.29	2.09	2.62	2.66	2.85	2.86	3.02	3.04	3.02
T_4	3.31	4.02	4.05	4.47	4.29	4.08	3.72	3.91	4.96
T_2	3.58	4.04	4.94	5.27	5.16	5.02	4.79	4.66	5.28
r-T_3	4.81	5.66	5.55	6.18	5.84	5.49	5.40	5.40	6.69

[a] Experimental changes in average free energies of binding were calculated from the data in Table 3, using eq ii. For each combination of hormone analogue and mutant receptor, predicted changes in the average free energies of binding were calculated using eq iii, adding the change in free energy resulting from the change in structure of ligand binding to wild-type receptor (column 2) plus the change in free energy from L-T_3 binding to mutant receptor (row 3). Those combinations showing significant deviations between those two values (≥0.6 kcal/mol) are underlined.

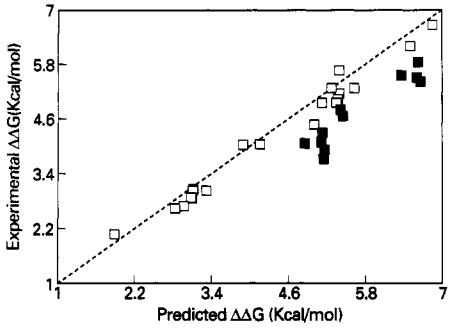

FIGURE 4: Plot of the experimental changes in free energies in the binding of thyroid hormone analogues to mutant receptors versus those predicted as the sum of the change from each analogue binding to wild-type receptor plus the change from L-T_3 binding to mutant receptor. The dashed line has a slope of unity, representing the expected values from additivity of these two variables, according to eq iii. Combinations showing significant deviations are shown as filled squares (■).

resistance to thyroid hormone. The data shown in Table 2 summarize the experimentally determined affinity constants. Alterations in the structure of either ligand or protein were found to produce sizable changes in the free energies of binding (see Table 3).

The free energy of binding of a ligand to the wild-type receptor (ΔG_{WT}) is the sum of the free energies of all these intermolecular interactions. Perturbation of one of these interactions by a single point mutation in the protein results in fairly small decreases in the measured free energies. For proteins which carry multiple mutations, measured free energy changes are usually the sum of the reductions measured in proteins carrying the separate, single mutations. However, in some cases, the measured change is found to differ from that predicted by this simple model. Such nonadditivity of the single mutant free energy changes implies that the mutated side chains interact with each other either by direct contact or through conformational perturbations (Wells, 1990). In like manner, nonadditivity of free energy changes produced by structural alterations in a protein and its ligand can be taken to indicate specific interactions (interplay) between the mutated single chain in the protein and the perturbed areas of the ligand. Again, the interplay may arise by direct contact or through conformational perturbation of the protein–ligand complex, induced by one change, resulting in altered interactions elsewhere. In the absence of crystallographic data, free energy analysis cannot distinguish between these two possibilities. Since all eight mutant forms of the receptor still have high affinities for L-T_3 (Table 2), it is unlikely that these sequence alterations result in drastic conformational changes in the structure of the hormone binding domain.

The present data could be interpreted using a structural model of the hormone binding domain recently proposed (McPhie *et al.*, 1993). It was suggested that the polypeptide chain of the hormone binding domain in h-TRβ1 is folded into an 8-fold α/β barrel with one extra α-helix in a loop (helix 4′; loop 4) on the amino-terminal face of the barrel (see Figure 5). Our prediction methods give no indication of the geometry of the loop regions, which may be flexible and may move to accommodate bound hormone. We suggest a model for the thyroid hormone binding site of h-TRβ1. Ionic bonds anchor the hormone's alanine side chain to loop 4 of an 8-fold α/β barrel. The phenyl ring lies across the amino-terminal face of the domain with the phenoxy ring pointing downward into the barrel and interacting with β-strand 8 on the opposite side. Loop 7 (the loop between helix 7 and β-strand 8) folds over the top of the barrel toward bound hormone. On this basis, we are in a position to discuss the locations and functions of the mutated residues in our structural model (Figure 5 and Table 1).

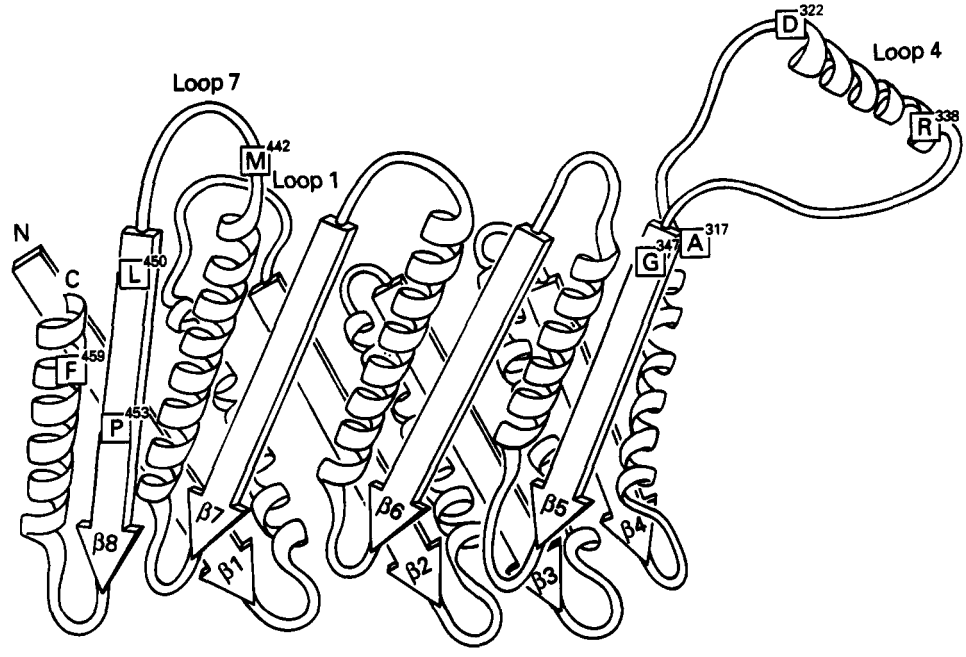

FIGURE 5: Schematic representation of our suggested structure of the hormone binding domain of h-TRβ1. The identity and approximate location of the naturally occurring point mutations (Refetoff, 1992) studied in the present work are shown in the single-letter amino acid code in the squares (see footnote 2). Sequence analysis and circular dichroism measurements on deletion mutants support the α/β barrel fold of the polypeptide chain which is traced as a ribbon. The numbers of β-strands are marked. The corresponding helices follow the β strands. No information is available on the positions or conformation of the loop regions. A typical α/β barrel domain is approximately 40 Å in diameter and 20 Å in depth (Branden & Tooze, 1991). The amino terminus and carboxyl terminus are denoted by N and C, respectively.

(1) Mutants IR (D322H) and KT (R338W) change the charge of conserved residues (Yaota *et al.*, 1990), which lie in a large hydrophilic loop, predicted to contain an extra α-helix (loop 4; helix 4′), between helix 4 and β-strand 5 of the α/β barrel. Other mutations that may alter the charge balance or the geometry of the loop are found in this region (Refetoff, 1992).

(2) Mutants ED (A317T) and NN (G347E) substitute large hydrophilic amino acids for small residues in hydrophobic areas on the amino-terminal face of the barrel at the top of helix 4 and strand 5, respectively.

(3) Mutant OK (M442V) decreases the size of a hydrophobic residue in a loop between helix 7 and β-strand 8.

(4) Mutants TP (L450H) and AH (P453H) change the hydrophobicity and conformation of β-strand 8 of the barrel. Other, similar mutants are found in this stretch of amino acids (Refetoff, 1992).

(5) Mutant RL (F459C) removes a large hydrophobic side chain, which binds helix 8 to β-strand 8, significantly altering the geometry of this strand and the adjoining regions of the barrel.

Substitutions at the 5′ position of the phenoxy ring will sterically hinder full insertion of ligands into the β barrel. In accordance with this prediction, mutants TP, AH, and RL, at residues predicted to be in the barrel, are not fully effective in reducing the binding of T_4 and r-T_3. For these analogues, nonadditivity of free energy changes is also seen for mutants OK and NN whose mutated residues are on the amino-terminal face of the barrel, indicating that the resultant distortion of the receptor–ligand complex extends to this region.

In contrast, the phenoxy ring of T_2 can still penetrate fully into the barrel, but its free energy of binding will be reduced by loss of specific interactions involving the 3′-iodine atom of L-T_3. Mutants TP and AH could accommodate T_2 effectively, whereas mutant RL interplays with this analogue. This may reflect direct interaction of the phenylalanine side chain (F^{459}) of the receptor with the 3′-iodine atom of L-T_3 [cf. Terry and Blake (1993)] or the distortion of the binding site as suggested above. T_2 also shows specific interplay with mutant OK (residue 442). Increased flexibility of the bound ligand may allow compensation for this structural defect in the protein.

To date, naturally occurring, clinically detected mutations have been reported at 22 different residues in the hormone binding domain (Refetoff, 1992; Sasaki *et al.*, 1992; Mixson *et al.*, 1992, 1993; Sakurai *et al.*, 1993; Geffner *et al.*, 1993). Only two of these lie outside the structural elements which we have located in the hormone binding site. A234T is predicted to lie in loop 1 (see Figure 5) between helix 1 and β-strand 2 of the barrel. Our model places loop 1 close to loop 7, both of which may fold toward the bound hormone molecule. Recent data indicate that hormone binding changes the sensitivity to proteolysis of a number of peptide bonds within this region (Bhat *et al.*, 1993). These observations support our suggestion of an interaction between loop 7 and the ligand. A final mutant, M310T, lies on the outside of the putative α/β barrel in helix 4. There is no indication of any direct participation of this residue in our postulated binding site. It may play a role in inhibition of ligand binding through conformational effects, which will only be revealed through high-resolution X-ray crystallographic analysis on the structure of this complex.

The eight-stranded α/β barrel is one of the most common motifs found in protein structures. Farber and Pestko (1990) consider the structure and evolution of α/β barrel enzymes. These all contain at least one large domain with this very stable fold, but can catalyze a wide variety of reactions. As they state, "the substrates for these enzymes have no similarities in size or functionality, and a wide range of cofactors is required". Specificity in these reactions is provided mainly by groups found in the highly variable loop regions between the secondary structure elements. This tertiary structure would be ideal in accommodating the wide variety of ligands bound by members of the steroid/retinoic acid/thyroid hormone receptor family. Using structural arguments, these

authors divided those α/β barrel proteins of known structure into four families, but could find no "conclusive proof of divergence as opposed to convergent evolution" among them. There is no definitive sequence homology among these protein families themselves, or with the thyroid hormone binding domain, so it may be that this stable structure has evolved independently several times. Members of two of these families also bind aromatic hydrophobic ligands (Farber & Pestko, 1990). Flavocytochrome β2, glycolate oxidase, and trimethylamine oxidase all bind flavin mononucleotide. *N*-(5-Phosphoribosyl)anthranilate isomerase, indole-3-glycerol-phosphate synthase, and tryptophan synthase α-subunit bind intermediates on the tryptophan biosynthetic pathways. In all cases, the aromatic compound is bound within the barrel, toward its carboxyl face. These proteins are all enzymes, and binding is partially determined by catalytic groups which lie in loops on the barrel's carboxyl face. In marked contrast, our model places the hormone binding site on the amino-terminal face, adjacent to the DNA binding domain. Such a location would facilitate regulation of the DNA binding properties of h-TRβ1, either through direct interaction of bound hormone with the DNA binding domain or through hormone-induced conformational changes in loops on this face of the hormone binding domain. Our putative model shares one characteristic in common with conventional α/β barrels; long flexible loops are associated with the binding site, whereas loops at the other face of the barrel are short (Wilmanns et al., 1991).

ADDED IN PROOF

While this paper was in review, an alternative model for the structure of the hormone binding domains of all members of the steroid/thyroid hormone/retinoic acid receptor family has been published (Goldstein *et al.*, 1993). The authors use a technique which maps the sequence of interest onto known protein crystal structures and judges the goodness-of-the-fit from a data base of the locations, physical properties, and structural preferences of the constituent amino acids. Their result is also an α/β structure, with many of the same secondary structural elements found in our model (McPhie et al., 1993). However, the selected tertiary structure is that of thermitase (1TEC); "an open twisted sheet of parallel β-strands, flanked on both sides by α-helices", yielding a very different configuration for the mutant residues which we discussed above. We considered and rejected this class of structures in our previous analysis, basing our arguments on the structure of other thyroid hormone binding sites. As Goldstein *et al.* point out, the precise lengths of these hormone binding domains are not known, but they give no clear rationalization for the sequences used in their analysis, which are all shorter than the usually accepted ones (Green & Chambon, 1986; Evans, 1988). In the case of h-TRβ1, we showed experimentally that all amino acids after Q205 are necessary for correct folding of the hormone binding domain (McPhie *et al.*, 1993). The model of Goldstein *et al.* begins at E268. It is difficult to see how these extra amino acids could be accommodated into their model.

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