Conformational Dynamics of Thyroid Hormones by Variable Temperature Nuclear Magnetic Resonance: The Role of Side Chain Rotations and Cisoid/Transoid Interconversions

Brendan M. Duggan[†] and David J. Craik*[,‡]

School of Pharmaceutical Chemistry, Victorian College of Pharmacy, Monash University, 381 Royal Parade, Parkville, Victoria 3052, Australia, and Centre for Drug Design and Development, University of Queensland, Brisbane, Queensland 4072, Australia

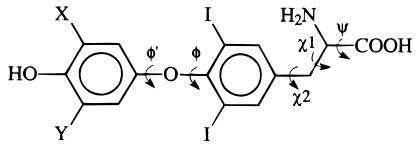
Received September 23, 1996[⊗]

^{1}H NMR spectra of the thyroid hormone thyroxine recorded at low temperature and high field show splitting into two peaks of the resonance due to the H2,6 protons of the inner (tyrosyl) ring. A single resonance is observed in 600 MHz spectra at temperatures above 185 K. An analysis of the line shape as a function of temperature shows that the coalescence phenomenon is due to an exchange process with a barrier of 37 kJ mol^{-1}. This is identical to the barrier for coalescence of the H2′,6′ protons of the outer (phenolic) ring reported previously for the thyroid hormones and their analogues. It is proposed that the separate peaks at low temperature are due to resonances for H2,6 in cisoid and transoid conformers which are populated in approximately equal populations. These two peaks are averaged resonances for the individual H2 and H6 protons. Conversion of cisoid to transoid forms can occur via rotation of either the alanyl side chain or the outer ring, from one face of the inner ring to the other. It is proposed that the latter process is the one responsible for the observed coalescence phenomenon. The barrier to rotation of the alanyl side chain is ≥37 kJ mol^{-1}, which is significantly larger than has previously been reported for Csp^2−Csp^3 bonds in other Ph-CH$_2$-X systems. The recent crystal structure of a hormone agonist bound to the ligand-binding domain of the rat thyroid hormone receptor (Wagner et al. *Nature* **1995**, *378*, 690−697) shows the transoid form to be the bound conformation. The significant energy barrier to cisoid/transoid interconversion determined in the current study combined with the tight fit of the hormone to its receptor suggests that interconversion between the forms cannot occur at the receptor site but that selection for the preferred bound form occurs from the 50% population of the transoid form in solution.

Introduction

Thyroid hormones regulate a wide range of metabolic processes as well as cell differentiation and growth. The major hormone secreted by the thyroid gland is thyroxine, usually referred to as T4 because of its four iodine substituents. Figure 1 shows that the iodines are attached to a diphenyl ether nucleus which is also substituted with a hydroxyl group and an alanyl side chain. In peripheral tissue deiodinase enzymes selectively remove one or more iodines to produce other thyroid hormones, including 3,5,3′-triiodothyronine (T3) and 3,5-diiodothyronine (T2). T3 is the most active of the hormones at the thyroid receptor located in cell nuclei.

There have been numerous studies of structure−activity relationships of the thyroid hormones (for a review, see Jorgensen[1]), and these have established the essential components for activity which include (i) a central hydrophobic nucleus composed of two mutually perpendicular aromatic rings linked by a bridging group, (ii) bulky lipophilic substituents ortho to the bridging group which maintain the active conformation of the aromatic moiety, (iii) a hydroxyl group para to the bridging group, and (iv) a charged side chain at the opposite end of the molecule to the hydroxyl group.



T4 X=I, Y=I
T3 X=I, Y=H
T2 X=H, Y=H

Figure 1. General structure of the thyroid hormones showing the torsion angles that define their conformation.

Until recently the molecular details of the interaction between the hormones and their receptor were not known. Most knowledge of thyroid hormone−protein interactions came from crystal structures of the hormones bound to one of the plasma transport proteins, transthyretin.[2−5] Crystal structures of T4 bound to transthyretin show that the hormone binds in a narrow channel and is completely surrounded by the protein. Very recently, knowledge of thyroid hormone−protein interactions has been expanded greatly with the determination of the crystal structure of a T3 analogue, 3,5-dimethyl-3′-isopropylthyronine, bound to the ligand-binding domain of the thyroid receptor.[6] The location of the hormone-binding site and the key interactions between receptor and ligand were identified in this study.

The crystal structures show that in both transthyretin and the thyroid receptor the hormones bind in a deep cavity lined with hydrophobic residues. Reaching such deeply buried sites would appear to require conformational flexibility in the hormone. There are few experimental methods for the study of such flexibility, and in the current study NMR methods are used to better

* To whom correspondence should be addressed. Phone: 61-7-3365 4945. Fax: 61-7-3365 2487. E-mail: d.craik@mailbox.uq.oz.au.
[†] Victorian College of Pharmacy.
[‡] University of Queensland.
[⊗] Abstract published in *Advance ACS Abstracts,* June 15, 1997.

S0022-2623(96)00661-9 CCC: $14.00

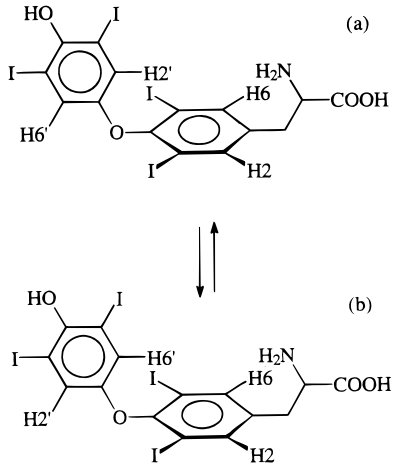


Figure 2. Interconversion of the distal and proximal protons of T4. (a) The H2′ proton is in the proximal position and the H6′ proton in the distal position. (b) Rotation about ϕ' by 180° has brought H6′ into the proximal position and H2′ into the distal position.

define the conformational dynamics of the thyroid hormones. It was of particular interest to examine dynamic motion associated with the inner (tyrosyl) ring, so that this could be compared with previous studies that focused mainly on the outer (phenolic) ring. Such studies are very important because the crystallographic studies of ligands bound to the thyroid receptor have implicated a structural role for the ligand which is of fundamental importance to the mechanism of action of the hormones. Thyroid hormones appear to control receptor function by inducing an active conformation of the receptor. Upon hormone binding, a conformational change takes place which directs the alignment of secondary structural elements of the receptor critical for function.[6]

Crystal structures of the thyroid hormones in the absence of the receptor show that the outer ring adopts a position roughly perpendicular to the inner ring in which steric clashes with the iodine atoms on the inner ring are minimized.[7] The outer ring may be on the same side of the inner ring as the alanyl side chain (the cisoid conformation), or it may be on the opposite side (the transoid conformation). In solution, the outer ring remains roughly perpendicular to the inner ring; however, it rotates so that the H2′ and H6′ protons exchange environments (Figure 2) on a microsecond time scale at room temperature, resulting in an averaged resonance for the two protons. At low temperature the rate of rotation is reduced and the resonances of the two protons are both observed, separated by 0.8 ppm. The outer ring proton which is proximal to the inner ring is upfield of the distal proton, due to a significant ring current shift. For T4, T3,[8] and several analogues[9,10] the barrier to the conformational exchange of H2′ and H6′ ranges from 33 to 38 kJ mol^{-1}.

When a series of variable temperature spectra was recorded to measure the energy barrier for rotation about the ether linkage of T4 and T3, an unexpected separation of the inner ring H2,6 resonance was observed,[8] in addition to the expected separation of the H2′,6′ resonance that had been reported earlier in some thyroid hormone analogues.[9] The origin of this phenomenon was not identified, but it suggests the presence of more complicated internal motions than simple rotation of the outer ring. Normally, it would be

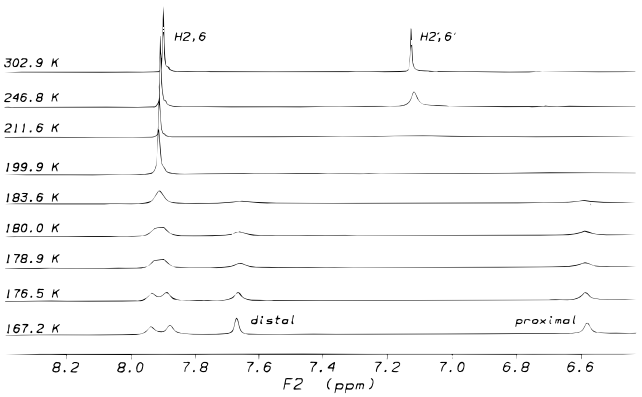


Figure 3. Stackplot of aromatic region of 600 MHz ^{1}H NMR spectra of thyroxine showing separation of both inner ring (H2,6) and outer ring (H2′,6′) resonances. The temperatures at which the spectra were obtained are noted just above each spectrum. Spectra are of a saturated solution of thyroxine (∼1 mM) in methanol-d_4.

expected that rapid rotation of the alanyl side chain would lead to averaging of the H2,6 signals at all accessible temperatures, but this may not be the case with thyroxine. To more fully understand the conformational dynamics of the thyroid hormones, a detailed study of the temperature dependence of the H2,6 resonances is undertaken in the current study.

Experimental Section

L-Thyroxine, L-thyroxine sodium salt, 3,5-diiodo-L-thyronine, and 3,5-diiodo-L-tyrosine were obtained from Sigma. Methanol-d_4, sodium deuteroxide, and deuterium chloride were from Cambridge Isotope Laboratories. Saturated solutions (∼1 mM) of the compounds in methanol were used. Spectra were recorded on a Bruker AMX-300 WB spectrometer at 300.13 MHz and a Bruker AMX-600 spectrometer at 600.13 MHz using a 90° pulse. Typical acquisition parameters were a 1.0 s relaxation delay, 3030 Hz spectral width (at 300 MHz), and 4096 data points. Boil off from a liquid nitrogen Dewar was used to lower the temperature. The actual probe temperature was determined from the chemical shift difference between the two resonances of a 100% methanol sample.[11] Spectra were processed using Felix 1.1 and imported into Microsoft Excel 5.0 to fit the theoretical spectra.

Results

Previous variable temperature studies at 300 and 400 MHz have shown that the H2′,6′ resonance of thyroxine observed at room temperature is an averaged signal resulting from the rapid exchange of proximal and distal environments and that the two protons yield separate signals at low temperature.[8] The focus in the previous study was on the barrier to rotation of the outer ring, which was determined from a line shape analysis of the coalescing H2′ and H6′ resonances, but at temperatures just above the freezing point of methanol, partial separation of the H2,6 inner ring resonances was also reported. The separation of a resonance due to ortho protons in a substituted benzene derivative is somewhat unusual and is further investigated in the current study. Low-temperature spectra were acquired at 600 MHz to determine the origin of the separation and to demonstrate reversible coalescence of the two signals.

Figure 3 shows the aromatic region of a series of 600 MHz ^{1}H NMR spectra of thyroxine. Above 240 K, both the H2,6 and H2′,6′ signals are singlets, but below 200 K, H2′,6′ broadens and separates into two peaks. The H2,6 resonance separates into two peaks below 180 K, but the separation of the two components is more than

Table 1. Coalescence Temperatures, Peak Separations, and Energy Barriers for Thyroxine

resonances	frequency (MHz)	T_c (K)	$\delta\nu$ (Hz)	$\Delta G^{\ddagger}$ (kJ mol^{-1})[a]
H2,6	300	176.6	18	37.1 ± 1.1
	600	182.4	35	37.3 ± 0.9
H2′,6′	300	197.5	324	36.9 ± 0.6
	600	201.1	652	36.4 ± 0.6

[a] Errors in $\Delta G^{\ddagger}$ were estimated by assuming an error in the coalescence temperature of ±1.5 K and in $\delta\nu$ of ±3 Hz.

1 order of magnitude smaller than for H2′,6′. These changes were reversible with an increase in temperature.

While the line shape changes of the H2′ and H6′ resonances are easily explained by rotation of the outer ring, the origin of the temperature dependence of the H2 and H6 resonances is less obvious. A preliminary inspection of the variable temperature spectra strongly suggests that an exchange process is responsible for the reversible spectral changes. However, because it is unusual for chemically inequivalent signals to be observed for ortho protons, it was important to eliminate other possible explanations. Given the small magnitude of the chemical shift difference between the resonances, the apparent coalescence could, in principle, be a result of the fortuitous temperature dependence of the chemical shifts. Changes in the chemical shifts which lead to partial overlap at lower temperatures could give the impression of coalescence. However, the experimental spectra show that once the separated peaks converge the combined peak sharpens (Figure 3), rather than separating again at higher temperature. Thus, the line shape changes are not due to simple temperature dependence of the chemical shifts in a nonexchanging system.

The existence of an exchange process was unequivocally confirmed by recording a second series of variable temperature NMR spectra at a different field strength (300 MHz) and noting a difference in coalescence temperature between the 300 and 600 MHz data. Table 1 summarizes the coalescence data for both the H2,6 and the H2′,6′ resonances and gives values for $\Delta G^{\ddagger}$ based on eq 1:[12]

$$\Delta G^{\ddagger} = 19.14 T_c \left[9.97 + \log\left(\frac{T_c}{\delta\nu}\right)\right] \qquad (1)$$

where T_c is the observed coalescence temperature, $\delta\nu$ is the chemical shift separation at low temperature, and $\Delta G^{\ddagger}$ is the energy barrier at the coalescence temperature.

The energy barriers determined using eq 1 are based on single measurements at the coalescence temperature. A more complete analysis of the data was made by fitting the complete set of variable temperature spectra at both fields to the line shape equation for a system undergoing two-site exchange:[13]

$$I = C\frac{\left(P\left(1 + \tau\left(\frac{P_B}{T_{2A}} + \frac{P_A}{T_{2B}}\right)\right) + QR\right)}{P^2 + R^2} \qquad (2)$$

Here I is the intensity of the NMR signal at frequency ν, C is a scaling factor, P_A and P_B are the populations of the two sites, k_A and k_B are the exchange rates between the sites, T_{2A} and T_{2B} are the transverse

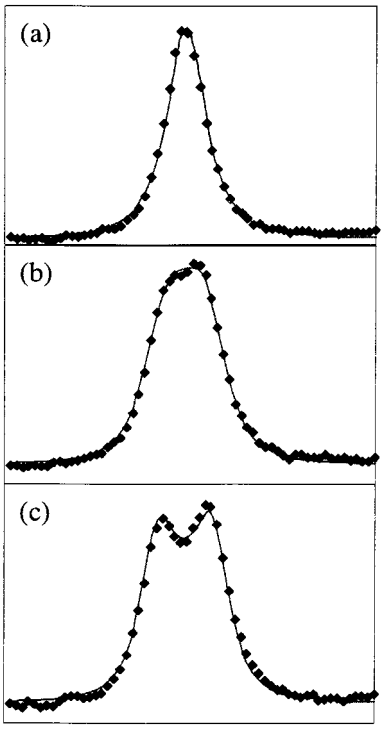


Figure 4. Fits of eq 2 to the 300 MHz data for the inner ring resonances of thyroxine. The filled diamonds (◆) represent the experimental points and the line the fitted theory. The data were acquired at temperatures close to the coalescence temperature: (a) 178.7, (b) 173.5, and (c) 171.5 K.

relaxation times of the two sites, and

$$P = \tau\left(\frac{1}{T_{2A}T_{2B}} - 4\pi^2\Delta\nu^2 + \pi^2\delta\nu^2\right) + \frac{P_A}{T_{2A}} + \frac{P_B}{T_{2B}}$$

$$Q = \tau(2\pi\Delta\nu - \pi\delta\nu(P_A - P_B))$$

$$R = 2\pi\Delta\nu\left(1 + \tau\left(\frac{1}{T_{2A}} + \frac{1}{T_{2B}}\right)\right) + \pi\delta\nu\tau\left(\frac{1}{T_{2B}} - \frac{1}{T_{2A}}\right) + \pi\delta\nu(P_A - P_B)$$

$$\tau = \frac{P_A}{k_B} = \frac{P_B}{k_A}$$

$$\delta\nu = \nu_A - \nu_B$$

$$\Delta\nu = \frac{\nu_A + \nu_B}{2} - \nu$$

Excellent fits are obtained, as seen from the examples shown in Figure 4. The exchange rates determined from these fits were used in the Eyring equation to obtain values for $\Delta G^{\ddagger}$, which were found to be in good agreement with the values obtained from the coalescence temperatures and peak separations.

Table 2 shows the derived thermodynamic parameters for the exchange process which is manifested by the variable temperature behavior of the H2,6 resonances. It is interesting to note that there is a slight population difference between the two conformations. This is apparent from the slight intensity difference of the separated H2,6 resonances seen in Figure 4. While the possibility of a minor impurity contributing to the intensity of one of the resonances is difficult to eliminate completely, if the intensity difference is intrinsic to the

Table 2. Parameters Derived from Line Shape Analysis of the H2,6 Resonances

parameter	300 MHz	600 MHz
P_A	0.49	0.49
P_B	0.51	0.51
$\delta\nu$ (Hz)	18	38
T_2 (s)	0.09	0.19
$\Delta G^{\ddagger}$ (kJ mol^{-1})[a]	37.2 ± 0.6	38.2 ± 0.6

[a] Errors in $\Delta G^{\ddagger}$ represent the standard deviation of fits to the Eyring equation of rate constants as a function of temperature obtained from eq 2.

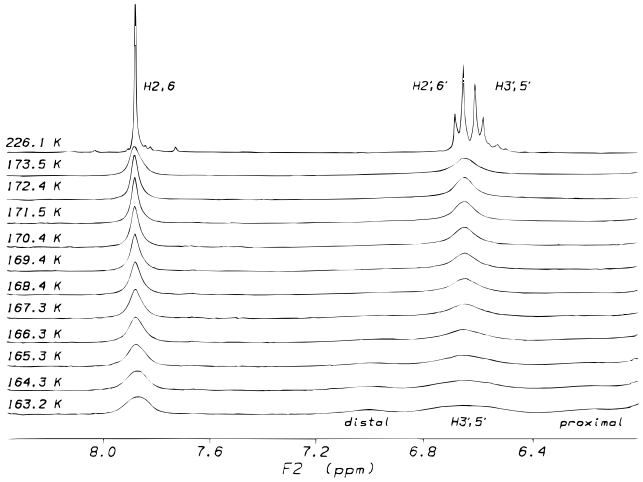


Figure 5. Stackplot of aromatic region of 300 MHz ^{1}H NMR spectra of 3,5-diiodothyronine. Note the separation of the outer ring resonances and the broadening of the inner ring resonances. Spectra are of a saturated solution of the free acid (~1 mM) in methanol. The temperatures at which the spectra were obtained are noted just above each spectrum.

exchange process then it suggests that the process is not a mutual interchange of H2 and H6 environments but involves energetically distinct conformers. To examine this possibility low-temperature spectra of analogues of thyroxine were recorded.

The effect of the outer ring was investigated by recording low-temperature spectra of 3,5-diiodothyronine (T2) and 3,5-diiodotyrosine. T2 differs from T4 in that the outer ring is not iodinated, whereas 3,5-diiodotyrosine does not have an outer ring at all. The H2′,6′ resonances of T2 separate at low temperature, and as the temperature is decreased further, the H2,6 resonance broadens suggesting that it too will separate at a sufficiently low temperature (Figure 5); however, the solution froze before such temperatures could be reached. The low-temperature spectra of 3,5-diiodotyrosine did not show any broadening of the H2,6 resonance over the temperatures examined (Figure 6). This suggests that the outer ring of the thyroid hormones is involved in the exchange process.

Low-temperature spectra of the sodium salt of thyroxine were recorded to determine if the introduction of charge to the side chain would influence the separation of the H2,6 resonance. It was found that this resonance from the sodium salt of thyroxine does not separate at low temperature. Only a single averaged resonance with no significant broadening was observed over the temperature range 162−236 K. A further series of experiments showed that the separation of the H2,6 resonance of the free acid could be removed by the addition of sodium deuteroxide and restored by the subsequent addition of deuterium chloride (Figure 7). This indicates that the charge state of the side chain

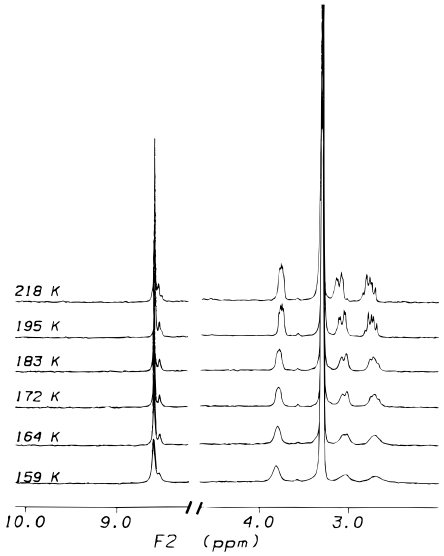


Figure 6. Stackplot of 600 MHz ^{1}H NMR spectra of 3,5-diiodotyrosine demonstrating the absence of any exchange phenomena. The temperatures at which the spectra were obtained are noted just above each spectrum. Spectra are of a saturated solution of the free acid (~1 mM) in methanol, and only the 3,5-diiodotyrosine resonances are shown.

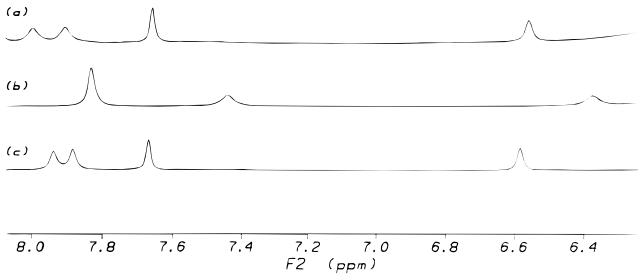


Figure 7. Stackplot of 600 MHz ^{1}H NMR spectra of thyroxine at 170.7 K showing the aromatic resonances: (a) saturated solution of thyroxine in methanol, (b) same sample after addition of NaOD, and (c) base-treated sample after the addition of DCl.

Table 3. Coalescence Temperatures, Peak Separations, and Energy Barriers to Exchange of Outer Ring Protons (H2′,6′) of T2 and T4 Sodium Salt

compd	frequency (MHz)	T_c (K)	$\delta\nu$ (Hz)	$\Delta G^{\ddagger}$ (kJ mol^{-1})[a]
T2	300	171.5	249	32.2 ± 0.7
T4·Na	600	182.0	615	32.9 ± 0.6

[a] Errors in $\Delta G^{\ddagger}$ were estimated by assuming an error in the coalescence temperature of ±1.5 K and in $\delta\nu$ of ±3 Hz.

significantly influences the detection of the exchange process. This could occur via the effect of the charge state on either the rate of the exchange process or the chemical shift separation of the two states.

By contrast with the lack of effect at H2,6, in both T2 and the sodium salt of T4, the conformational exchange process affecting the outer ring was detected via a separation of the H2′,6′ resonances. Energy barriers were determined from the coalescence temperatures and peak separations using eq 1 and are reported in Table 3. These are of similar magnitude to the values for the free acid of T4.

Discussion

Having established that the separation of the H2,6 resonances at low temperature is the result of an exchange process, it is now of interest to identify the

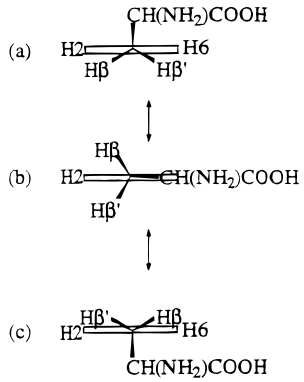


Figure 8. Rotation about $\chi 2$ exchanges the local environments of H2 and H6. In exchanging between one minimum energy conformation (a) and the other (c), the molecules pass through the maximum energy conformation (b). Rotations about Csp^2-Csp^3 bonds are associated with a predominantly 2-fold symmetry, and there is general agreement that the perpendicular form a or c is of lower energy than form b.[18]

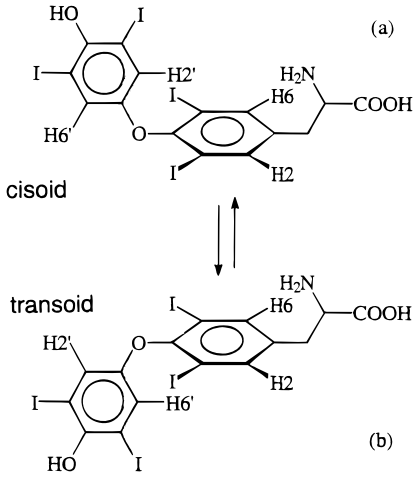


Figure 9. Interconversion of the cisoid (a) and transoid (b) forms of thyroxine by concerted rotation of ϕ and ϕ'. In the cisoid form the positioning of the outer ring and the alanyl side chain on the same face of the inner ring creates a different environment for H2 and H6, compared with the transoid conformation.

nature of the process. The possibilities for assignment of the separate low-temperature resonances can be seen from a consideration of the conformation of T4 shown in Figure 2. One explanation for the separate resonances is that one is due to H2 and one to H6. In principle their environments can be different due to either asymmetry of the alanyl side chain or orientation of the outer ring. In the X-ray structure of T4[7] the alanyl side chain prefers a conformation in which the $C\alpha-C\beta$ bond is roughly perpendicular to the aromatic ring ($\chi 2 = 90°$) (Figure 8a,c). In such a conformation H2 and H6 are in different chemical environments and potentially have different chemical shifts because of the asymmetry of the alanyl side chain. This inequality is not removed by rapid rotation about $\chi 1$ (i.e., the CH_2-CH bond) because of the chiral center at $C\alpha$. (The lack of averaging due to rotation of adjacent chiral groups is well known and most usually observed for β-methylene protons, as indeed it is for T4, where $H\beta$ and $H\beta'$ have a chemical shift difference of 0.24 ppm despite the fact that rotation about $\chi 1$ is likely to be rapid.) By contrast, rotation about $\chi 2$ by 180° (i.e., Figure 8a ↔ c) results in an exchange of the local environment of H2 and H6 with respect to the alanyl side chain, ignoring possible effects from the outer ring for the moment. Rapid rotation (i.e., at a rate faster than the chemical shift difference) would produce a single averaged resonance for H2 and H6, while slow rotation would produce separate resonances. The hypothesis to be presented below is that such a process does indeed occur but that this is not the reason for the observed coalescence phenomenon.

The crystal structures of many of the thyroid hormones exist in a twist−skewed conformation (i.e., ϕ' is displaced slightly from zero so that the two aromatic rings are not quite perpendicular),[7] which provides another possible mechanism for inequality of H2 and H6. A simple calculation based on ring current effects[14] suggests that the maximum possible chemical shift difference of the H2 and H6 protons in such a twist−skewed conformation is 0.4 ppm, and this is achieved when $\phi' = \pm 30°$. However, this is not likely to be the factor responsible for the coalescence process observed in solution because interchange between symmetry-related skewed forms in solution is extremely rapid (as demonstrated by ^{13}C T_1 measurements[15]) and will result in an averaged resonance for H2 and H6 at all accessible temperatures.

Another way in which the outer ring could contribute to inequivalence of H2 and H6 is due to its role, together with the alanyl side chain, in producing cisoid and transoid forms of the hormone (Figure 9). In the cisoid conformation H2 and H6 are in a different environment to those of H2 and H6 in the transoid conformation. The different environments are produced as a result of the differing relative orientation of the outer ring and alanyl side chain, as illustrated in Figure 9. For convenience we shall use the suffixes "c" and "t" to refer to the cisoid and transoid environments, respectively. Consideration of Figure 9 shows that movement of the outer ring from one face of the inner ring to the opposite face (likely to occur by concerted rotation of ϕ and ϕ' [16]) interchanges H2c with H2t and H6c with H6t. Rotation about $\chi 2$ by 180° interchanges H2c with H6t and H6c with H2t, whereas rotation of both $\chi 2$ and ϕ by 180° interchanges H2 with H6 in both the cisoid and transoid forms.

The hypothesis presented here is that the observed variable temperature behavior reflects the interconversion of cisoid and transoid conformers. It is proposed that any shift difference between H2 and H6 in either cisoid or transoid forms (due to asymmetry of the alanyl side chain) is sufficiently small that only an averaged resonance is observed, even at the lowest temperature studied. Thus, the peaks observed at 7.94 and 7.88 ppm in the 170.7 K spectra of thyroxine are due to H2,6c and H2,6t rather than to H2c and H6c or H2t and H6t, i.e., the observed two peaks are averaged resonances for H2,6: one peak for the transoid pair of protons and one peak for the cisoid pair. If the temperature could be lowered further than was achievable in this study, then it may be possible to separate each of these resonances so that all four resonances, H2c, H6c, H2t, and H6t, could be observed.

The fact that no inequality is observed in the 3,5-diiodotyrosine spectra (Figure 6), where there is no outer ring, and hence no possibility of cisoid and transoid conformers, provides support for the above interpretation of the low-temperature spectra. Further support comes from the intensity of the separated peaks in the low-temperature spectra of thyroxine which show the

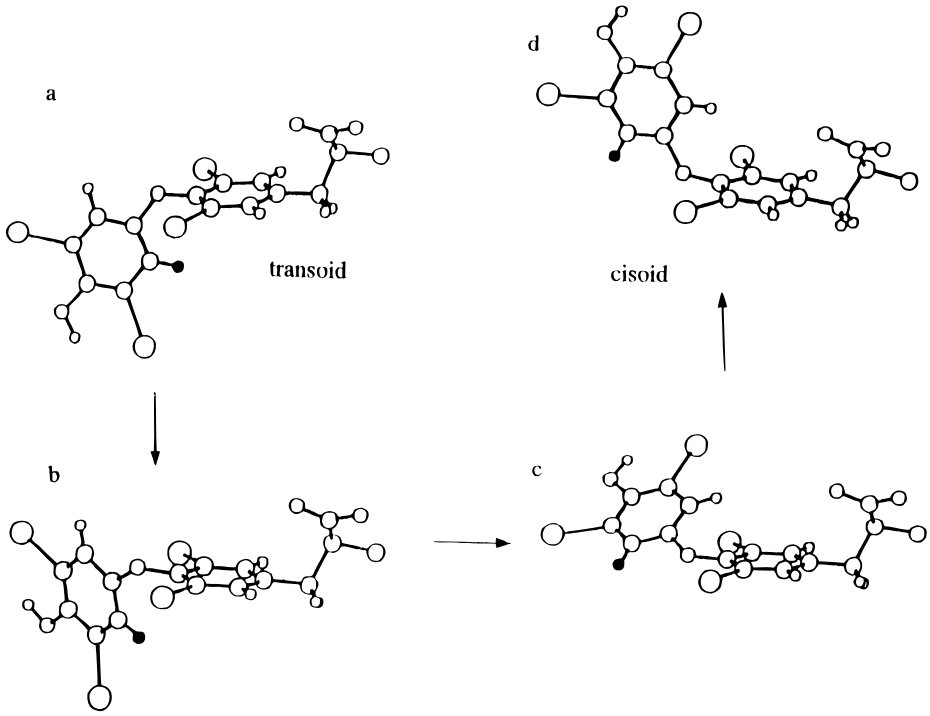


Figure 10. Thyroxine structures illustrating the interconversion of H2′ and H6′ via concerted rotation of torsion angles ϕ and ϕ' of the diphenyl ether moiety. As well as interconverting the environments of H2′ and H6′, this process interchanges the cisoid and transoid forms. The transoid form is shown in panel a. Intermediate forms b and c involve cooperative rotations of ϕ and ϕ' to minimize steric classes, leading eventually to the cisoid form d. The outer ring H6′ proton is shaded to indicate its transition from a proximal environment in form a to a distal environment in form d.

upfield resonance to be more intense than the downfield one. Slightly different populations of the cisoid and transoid conformers could easily account for the intensity difference. The intensity difference cannot be explained if the two low-temperature resonances are assumed to arise from separate peaks for H2 and H6 due to asymmetry of the side chain. Nevertheless the possibility of an impurity under the more intense resonance, combined with the coalescence phenomenon for H2,6 arising from a rotational barrier about $\chi 2$ being coincidently similar to that for outer ring rotation, cannot be completely excluded.

Close inspection of the proximal and distal resonances from the outer ring in low-temperature spectra (Figure 3) also supports the cisoid/transoid hypothesis. The proximal resonance is expected to be more sensitive to the difference between cisoid and transoid conformers than the distal resonance and could conceivably separate into two peaks: a proximal-cisoid resonance and a proximal-transoid resonance. The broadening of the proximal resonance (but not the distal one) suggests this may occur at temperatures just below the lowest temperature reached in this work.

The populations of the cisoid and transoid forms derived from the curve fitting of the low-temperature spectra were 49% and 51%, indicating that the two conformers are of nearly equivalent energy. It is not possible to determine which conformer has the slightly higher population. In the crystal state the proportions of the two conformers are similar too. Of 21 different structures of thyroid hormones and analogues, 10 are in the cisoid form and 11 in the transoid.[7]

In previous studies[8] it has been established that the barrier to interconversion of H2′ and H6′ is 36 kJ mol^{-1} and that the likely mechanism by which this occurs is concerted rotation of ϕ and ϕ', as illustrated in Figure 10.[16] A consequence of such a motion is that it interconverts cisoid and transoid forms, if the conformation of the alanyl side chain remains fixed. Because the barrier to interconversion of cisoid and transoid forms detected in the current study (as determined from the coalescence of the H2 and H6 resonances) is the same (within experimental error) as that for interconversion of distal and proximal protons (see Table 1), it is likely that it is movement of the outer ring that is responsible for the H2,6 coalescence, and therefore the barrier to rotation of the side chain must be equal to, or greater than, the barrier for rotation of the outer ring. If the barrier to rotation of the side chain were less than the 37 kJ mol^{-1} required to rotate the outer ring, then a smaller value for the energy barrier would have been determined from the coalescence of the H2,6 resonances. If the barrier to rotation of the side chain were larger than the barrier to rotation of the outer ring, then the detected barrier would still be 37 kJ mol^{-1}, as outer ring motion would provide a low-energy route to cisoid/transoid interconversion. In the unlikely event that H2′ to H6′ interconversion occurs via rotation about ϕ' only, in which case no cisoid to transoid interconversion results, then the barrier to rotation of the side chain must be exactly 37 kJ mol^{-1}. In summary, the barrier for side chain rotation must be $\geq$37 kJ mol^{-1}.

This value is substantially larger than the barrier to rotation about other Csp^2-Csp^3 bonds in substituted phenyl derivatives. Barriers for rotation about the Csp^2-Csp^3 bond of a range of Ph-CH_2-X compounds have been calculated[17] and determined experimentally[18] as ranging between 5 and 21 kJ mol^{-1}. The reason for the much larger barrier in T4 is not clear, although it may in part reflect the steric bulk of the side chain. In a series of benzyl halides the barrier increased from 9 to 21 kJ mol^{-1} in the order Cl < Br < I.[19] However, the benzyl iodide barrier is still a factor of 2 smaller than the barrier shown by T4, despite the similar steric

bulk of the side chains. The benzyl iodide barrier was measured in nematic solvents,[19] but the barrier to rotation about the Csp^2-Csp^3 bond in a range of Ph-CH_2-X molecules is only marginally dependent on solvent,[18] so the use of methanol as solvent is not likely to increase the barrier by a factor of 2−4 to bring it to the magnitude of that detected experimentally in this study.

Given that the barrier to side chain rotation must be greater than or equal to 37 kJ mol^{-1}, it is possible to estimate an upper limit for the chemical shift separation of H2 and H6. Substitution of this barrier into eq 1 for the lowest temperature examined (170.7 K) shows that separate peaks would be observed if the H2/H6 separation was ∼8 Hz at 600 MHz. No sign of further splitting of the two H2,6 peaks (i.e., H2,6c and H2,6t) is observed at this temperature, so the chemical shift difference between H2 and H6 must be substantially less than 0.01 ppm. If the barrier to side chain rotation is any higher than 37 kJ mol^{-1}, then an even smaller value for the chemical shift separation of the H2 and H6 resonances would be predicted. Thus, it can be safely concluded that the shift separation of the H2 and H6 environments (in either cisoid or transoid form) is ≤0.01 ppm. This estimate is consistent with expectations based on the fact that this separation arises from the chirality of the side chain $CH(NH_2)COOH$ group. This group produces a shift difference of 0.24 ppm in the adjacent Hβ,β' protons, and so the effect two bonds further away at H2 and H6 would be expected to be substantially smaller.

The findings that both cisoid and transoid forms are significantly populated in solution and the fact that there is a significant barrier to side chain rotation have some implications for the binding of thyroid hormones to their receptor. The crystal structure of the ligand-binding domain of the thyroid receptor complexed with a hormone analogue shows that the thyroid hormones are likely to bind to the receptor in the transoid conformation.[6] The hormone agonist 3,5-dimethyl-3′-isopropylthyronine is oriented in the receptor such that the outer ring enters the binding cavity first and is tightly packed in a hydrophobic pocket. The inner ring is also deeply buried in the hydrophobic core of the receptor protein, and there is thus no opportunity for conformational interconversion of the diphenyl ether moiety at the bound site. This does not exclude the possibility of small amplitude conformational flexibility of the hormone as it enters the binding pocket, and indeed, it appears that some degree of conformational flexibility is likely to be necessary to reach such a deeply buried site. By contrast with the diphenyl ether moiety, the alanyl side chain of the hormone agonist binds in a much looser polar pocket of the receptor; however, there still appears to be insufficient room for large scale motions of this side chain at the bound site.

The relatively large barrier to cisoid/transoid interconversion in the free state (via either diphenyl ether or side chain rotation) combined with the relatively tight packing of the hormone in the bound state likely precludes the possibility of cisoid/transoid interconversions occurring in the bound state. Selection for the preferred transoid conformer thus occurs from the approximate 50% population of this form in solution. Initial loose binding of the cisoid form with subsequent conversion to transoid during the binding process appears unlikely because the relatively high barrier determined here corresponds to a microsecond time scale for the interconversion at physiological temperature. The high binding affinity of thyroid hormones for the receptor precludes a slow initial binding step. There is significant therapeutic potential for a thyroid hormone antagonist, and the current study, combined with information on the bound conformation,[6] suggests that significantly improved binding affinity to the receptor could be achieved by the design of analogues with a preferred transoid conformation.

Acknowledgment. This work was supported in part by a grant from the Australian Research Council. D.J.C. is an ARC Senior Fellow. We thank Dr. T. Schaefer for helpful comments.

References

(1) Jorgensen, E. C. Thyroid Hormones and Analogs. II. Structure-Activity Relationships. In *Hormonal Proteins and Peptides*, 1st ed.; Li, C. H., Ed.; Academic Press: New York, 1978; Vol. 6, pp 107−204.
(2) Blake, C. C. F.; Oatley, S. J. Protein-DNA and Protein-Hormone Interactions in Prealbumin: A Model of the Thyroid Hormone Nuclear Receptor? *Nature* **1977**, *268*, 115−120.
(3) Oatley, S. J.; Blake, C. C. F.; Burridge, J. M.; de la Paz, P. The Structure of Human Serum Prealbumin and the Nature of its Interactions with the Thyroid Hormones. In *X-Ray Crystallography and Drug Action*; Horn, A. S., DeRanter, C. J., Eds.; Clarendon Press: Oxford, 1984; pp 207−221.
(4) Wojtczak, A.; Luft, J.; Cody, V. Mechanism of Molecular Recognition: Structural Aspects of 3,3′-Diiodo-L-thyronine Binding to Human Serum Transthyretin. *J. Biol. Chem.* **1992**, *267*, 353−357.
(5) de la Paz, P.; Burridge, J. M.; Oatley, S. J.; Blake, C. C. F. Multiple Modes of Binding of Thyroid Hormones and Other Iodothyronines to Human Plasma Transthyretin. In *The Design of Drugs to Macromolecular Targets*; Beddell, C. R., Ed.; John Wiley and Sons: Chichester, 1992; pp 119−172.
(6) Wagner, R. L.; Apriletti, J. W.; McGrath, M. E.; West, B. L.; Baxter, J. D.; Fletterick, D. J. A Structural Role for Hormone in the Thyroid Hormone Receptor. *Nature* **1995**, *378*, 690−697.
(7) Cody, V. Thyroid Hormone Interactions: Molecular Conformation, Protein Binding, and Hormone Action. *Endocr. Rev.* **1980**, *1*, 140−166.
(8) Gale, D. J.; Craik, D. J.; Brownlee, R. T. C. Variable Temperature NMR Studies of Thyroid Hormone Conformations. *Magn. Reson. Chem.* **1988**, *26*, 275−280.
(9) Emmett, J. C.; Pepper, E. S. Conformation of Thyroid Hormone Analogues. *Nature* **1975**, *257*, 334−336.
(10) Mazzocchi, P. H.; Ammon, H. L.; Colicelli, E.; Hohokabe, Y.; Cody, V. NMR Studies of Triiodothyropropionic Acid in Ethanol-HCl. *Experientia* **1976**, *32*, 419−420.
(11) Kaplan, M. L.; Bovey, F. A.; Cheng, H. N. Simplified Method of Calibrating Thermometric Nuclear Magnetic Resonance Standards. *Anal. Chem.* **1975**, *47*, 1703−1705.
(12) Günther, H. *NMR Spectroscopy, An Introduction*; John Wiley & Sons: New York, 1980.
(13) Sandström, J. *Dynamic NMR Spectroscopy*; Academic Press: London, 1982.
(14) Becker, E. D. *High Resolution NMR*; Academic Press: New York, 1980.
(15) Duggan, B. M.; Craik, D. J. ^1H and ^{13}C NMR Relaxation Studies of Molecular Dynamics of the Thyroid Hormones Thyroxine, 3,5,3′-Triiodothyronine, and 3,5-Diiodothyronine. *J. Med. Chem.* **1996**, *39*, 4007−4016.
(16) Craik, D. J.; Andrews, P. R.; Border, C.; Munro, S. L. A. Conformational Studies of Thyroid Hormones. I The Diphenyl Ether Moiety. *Aust. J. Chem.* **1990**, *43*, 923−936.
(17) Hehre, W. J.; Radom, L.; Pople, J. A. Molecular Orbital Theory of the Electronic Structure of Organic Compounds. XII. Conformations, Stabilities, and Charge Distributions in Monosubstituted Benzenes. *J. Am. Chem. Soc.* **1972**, *94*, 1496−1504.
(18) Schaefer, T.; Hazendonk, P.; McKinnon, D. M. ^1H, ^{19}F, and ^{13}C Nuclear Magnetic Resonance Approaches to the Barrier to Rotation about the Csp^2-Csp^3 bond in 1,1,1-trifluoro-2-phenylethane. Proximate Coupling Constants and Molecular Orbital Computations. *Can. J. Chem.* **1995**, *73*, 1387−1394.
(19) Celebre, G.; DeLuca, G.; Longeri, M.; Ferrarini, A. Investigation of Internal and External Potentials Acting on Benzyl Halides Dissolved in Different Nematic Solvents. *Mol. Phys.* **1994**, *83*, 309−326.

JM9606610