Thyroxine-Protein Interactions

INTERACTION OF THYROXINE AND TRIIODOTHYRONINE WITH HUMAN THYROXINE-BINDING GLOBULIN*

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The effect of temperature on the binding of thyroxine and triiodothyronine to thyroxine-binding globulin has been studied by equilibrium dialysis. Inclusion of ovalbumin in the dialysis mixture stabilized thyroxine-binding globulin against losses in binding activity which had been found to occur during equilibrium dialysis. Ovalbumin by itself bound the thyroid hormones very weakly and its binding could be neglected when analyzing the experimental results. At pH 7.4 and 37° in 0.06 M potassium phosphate/0.7 mM EDTA buffer, thyroxine was bound to thyroxine-binding globulin at a single binding site with apparent association constants: at 5°, K = 4.73 ± 0.38 x 10^4 M^-1; at 25°, K = 1.55 ± 0.17 x 10^4 M^-1; and at 37°, K = 9.08 ± 0.62 x 10^4 M^-1.

Scatchard plots of the binding data for triiodothyronine indicated that the binding of this compound to thyroxine-binding globulin was more complex than that found for thyroxine. The data for triiodothyronine binding could be fitted by assuming the existence of two different classes of binding sites. At 5° and pH 7.4 nonlinear regression analysis of the data yielded the values n1 = 1.04 ± 0.10, K1 = 3.35 ± 0.63 x 10^4 M^-1 and n2 = 1.40 ± 0.08, K2 = 0.69 ± 0.20 x 10^4 M^-1. At 25°, the values for the binding constants were n1 = 1.04 ± 0.38, K1 = 6.5 ± 2.8 x 10^4 M^-1 and n2 = 0.77 ± 0.22, K2 = 0.43 ± 0.62 x 10^4 M^-1. At 37°, where less curvature was observed, the estimated binding constants were n1 = 1.02 ± 0.06, K1 = 4.32 ± 0.59 x 10^4 M^-1 and n2K2 = 0.056 ± 0.012 x 10^4 M^-1. When n1 was fixed at 1, the resulting values obtained for the other three binding constants were at 25°, K1 = 6.12 ± 0.35 x 10^4 M^-1, n2 = 0.72 ± 0.18, K2 = 0.73 ± 0.36 x 10^4 M^-1; at 37°, K1 = 3.80 ± 0.22 x 10^4 M^-1, n2 = 0.44 ± 0.22, and K2 = 0.43 ± 0.38 x 10^4 M^-1. The thermodynamic values for thyroxine binding to thyroxine-binding globulin at 37° and pH 7.4 were ΔG° = -14.1 kcal/mole, ΔH° = -8.96 kcal/mole, and ΔS° = +16.7 cal degree^-1 mole^-1. For triiodothyronine at 37°, the thermodynamic values for binding at the primary binding site were ΔG° = -12.8 kcal/mole, ΔH° = -11.9 kcal/mole, and ΔS° = +1.4 cal degree^-1 mole^-1.

Measurement of the pH dependence of binding indicated that both thyroxine and triiodothyronine were bound maximally in the region of physiological pH, pH 6.8 to 7.7.

Thyroxine-binding globulin is physiologically the most important carrier of the thyroid hormones in human blood (1, 2). Thyroxine is relatively tightly bound to thyroxine-binding globulin with an estimated association constant of the order of 10^19 M^-1 based on indirect measurements in human sera (1) and as determined by ultrafiltration studies with the purified protein (3). Less information is available on the binding characteristics of triiodothyronine, the more active form of the hormone. In this paper we describe binding data for the interaction of thyroxine and triiodothyronine with thyroxine-binding globulin at different temperatures as determined by equilibrium dialysis. The effect of pH on binding has also been determined.

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EXPERIMENTAL PROCEDURE

Fresh frozen human plasma was obtained from the New York Blood Center through the courtesy of Dr. A. R. Neurath.

Thyroxine-binding globulin was isolated from fresh frozen plasma by the three-step procedure described previously (4), consisting of affinity chromatography on thyroxine-Sepharose, chromatography on DEAE-Sephadex A-50, and preparative electrophoresis in polyacrylamide gel. For preparative electrophoresis in polyacrylamide gel a Buchler Poly-Prep 200 apparatus was used. Usually the pooled plasma of six individuals, about 1500 ml, was taken as starting material. Fractions containing thyroxine-binding globulin were, however, not frozen at 20° after each step as had been done previously, but were stored at 4° in the buffer used for elution. The purification procedure was completed in about 10 days. The final purified thyroxine-binding globulin product obtained after preparative polyacrylamide gel electrophoresis was kept at 4° in the elution buffer, 0.05 M Tris-HCl, pH 8.2 to 8.3. In some cases, sodium azide (0.025% final concentration), was added as preservative to the protein solution. Under these conditions of storage, that is, at 4° rather than at -20°, no loss of thyroxine-binding activity was observed over a period of about 8 weeks. When stored at
-20°C, losses of up to 20% in binding activity have been observed after a single thawing of dilute thyroxine-binding globulin solutions (0.2 to 0.5 µg/ml).

\[ ^{131}I \text{-labeled thyroxine and triiodothyronine in 50% propylene glycol containing 2 mg/ml of cysteine hydrochloride as preservative were obtained from Abbott Laboratories. Inorganic } \left[^{131}I\right] \text{iodide, was purchased from the Union Carbide Corp., Tuxedo, N.Y. Inorganic iodide contamination of the } \left[^{131}I\right] \text{-labeled thyroid hormone preparations ranged from 1 to 5%. The extent of inorganic iodide contamination was determined both by paper electrophoresis (5) and by chromatography of the } \left[^{131}I\right]\text{iodothyronine compounds on paper in 1-butanol/dioxane/2 N ammonia (4/1/5, v/v). Dialysis mixtures containing } \left[^{131}I\right]\text{-thyroxine were also analyzed directly for inorganic iodide by the trichloroacetic acid precipitation method described by Oppenheimer et al. (5, 6). All three methods agreed to within ±10%}. An accurate determination of iodide is essential since thyroxine-binding globulin does not bind iodide, whereas the thyroid hormones are bound very tightly. The interaction of inorganic iodide with thyroxine-binding globulin was studied directly by equilibrium dialysis with trace quantities of \(^{131}I\)iodide. \(^{131}I\)Triiodothyronine solutions contained less than 2% thyroxine as contaminant as ascertained by paper chromatography.

Carrier thyroxine and triiodothyronine were obtained from Aldrich Chemical Co.

The concentration of thyroid hormones in stock solutions was determined by measurement of the absorbance at 325 nm for thyroxine and 300 nm for triiodothyronine. At these wavelengths, the molar absorptions in 0.01 M NaOH for thyroxine and triiodothyronine were taken as 6.21 x 10\(^4\) and 4.66 x 10\(^4\) M\(^{-1}\) cm\(^{-1}\), respectively.

Buffers used in equilibrium dialysis were prepared by mixing 0.06 M NaH\(_2\)PO\(_4\) (pH 8.6), Tris-HCl (pH 8.8 to 11.0), and EDTA to give a final pH of 7.4, the buffer mixture consisted of 0.06 M potassium phosphate buffer, pH 7.4, the buffer mixture was 0.06 M concentration and containing 0.7 M metal ion impurities. Protein concentrations ranged from 0.5 to 2 mg/ml was added to the mixture and slow shaking was used, 40 to 50 strokes/min.

The data for thyroxine binding in the presence and absence of ovalbumin at 37°C and pH 7.4 are shown in Fig. 1. There was much stronger binding of thyroxine to the globulin in the presence of ovalbumin. Ovalbumin by itself bound the thyroid hormones very weakly with an estimated association constant in the range of \(1 \times 10^3\) M\(^{-1}\) and thus its binding could be neglected when analyzing the data. Consequently, with the exceptions noted below, equilibrium dialysis at all temperatures was done routinely in the presence of ovalbumin (1 mg/ml).

Scatchard plots for the binding of thyroxine to thyroxine-binding globulin at 5°C and 25°C are given in Fig. 2. The binding data (Figs. 1 and 2) fall close to a straight line in agreement with Equation 1. A low level of residual binding of thyroxine was also observed at \(v\) values above 1.1 (Figs. 1 and 2) but this was not considered to be significant. Regression lines were calculated using the results for binding at \(v\) values below 1. Based on the value of the intercept on the \(y\) axis (n) and the slope (K) of each line the estimated binding constants and standard errors were: at 37°C (Fig. 1) \(n = 1.07 ± 0.07, K = 9.08 ± 0.62 \times 10^3\) M\(^{-1}\); at 25°C (Fig. 2) \(n = 1.12 ± 0.08, K = 1.55 ± 0.17 \times 10^4\) M\(^{-1}\); and at 5°C (Fig. 2) \(n = 0.92 ± 0.07, K = 4.73 ± 0.38 \times 10^4\) M\(^{-1}\).

**RESULTS**

In preliminary studies it was found that the binding of thyroxine to thyroxine-binding globulin was greatly affected by the rate of shaking of the equilibrium dialysis mixtures. For example, in one experiment performed at 5°C, shaking at about 200 strokes/min over a period of 48 hours resulted in almost complete loss of binding activity at a protein concentration of 1 µg/ml. Apparently thyroxine-binding globulin is very sensitive to surface denaturation. In an attempt to stabilize the binding protein during dialysis, ovalbumin at a concentration of 1 mg/ml was added to the mixture.

**Scatchard plots for the binding of thyroid hormones to thyroxine-binding globulin at 5°C and 25°C are given in Fig. 2.**

**Fig. 1. Interaction of thyroxine with thyroxine-binding globulin at 37°C and pH 7.4 in 0.06 M potassium phosphate/0.7 mM EDTA buffer.**

Binding in the presence (O) and absence (O) of ovalbumin. Ovalbumin by itself bound the thyroid hormones very weakly with an estimated association constant in the range of \(1 \times 10^3\) M\(^{-1}\) and thus its binding could be neglected when analyzing the data. Consequently, with the exceptions noted below, equilibrium dialysis at all temperatures was done routinely in the presence of ovalbumin (1 mg/ml).

The initial protein concentration was 1.67 µM and the starting volume was 1 ml. Thyroxine solutions were added by hand in 5-µl volumes. The readings were corrected for dilution and attenuation of fluorescence arising from absorption of ultraviolet light by thyroxine.
Fluorimetric titration of the thyroxine-binding globulin with thyroxine at 25° and pH 7.4 gave the result shown in Fig. 3. Maximal quenching of fluorescence of the protein was observed at an average mole ratio of thyroxine to protein of 0.86, as estimated from the intersection of the two lines in Fig. 3.

Binding of Triiodothyronine—As shown in Fig. 4, the data for binding of triiodothyronine at 5° deviated from linearity. The data could be fitted by assuming the existence of two classes of independent binding sites as given by Equation 2.

\[
\frac{n_1}{1 + K_1 + K_2} + \frac{n_2}{1 + K_1 + K_2} = \frac{n}{1 + K_1 + K_2}
\]

Nonlinear regression analysis of the binding data for triiodothyronine at 5° according to Equation 2 gave values for the binding parameters, \(n_1 = 1.04 \pm 0.10, K_1 = 3.35 \pm 0.63 \times 10^8 \text{ M}^{-1}\) and \(n_2 = 1.40 \pm 0.08, K_2 = 0.69 \pm 0.20 \times 10^8 \text{ M}^{-1}\). The results obtained for triiodothyronine binding at 25° and 37° are shown in Fig. 5. At 25°, nonlinear regression analysis of the data yielded the values, \(n_1 = 1.04 \pm 0.38, K_1 = 6.5 \pm 2.8 \times 10^8 \text{ M}^{-1}\) and \(n_2 = 0.77 \pm 0.22, K_2 = 0.43 \pm 0.62 \times 10^8 \text{ M}^{-1}\). At 37°, where less curvature was observed, the estimated binding constants were \(n_1 = 1.02 \pm 0.08, K_1 = 4.32 \pm 0.59 \times 10^8 \text{ M}^{-1}\) and \(n_2 = 1.07 \pm 0.06, K_2 = 0.056 \pm 0.012 \times 10^8 \text{ M}^{-1}\). It was possible to obtain separate values for the secondary binding constants at 37° by fixing \(n_1\) at 1 and solving for the other three binding parameters. In this case, assuming \(n_1 = 1\), then at 37°, \(K_1 = 3.80 \pm 0.22 \times 10^8 \text{ M}^{-1}\) and \(K_2 = 0.43 \pm 0.38 \times 10^8 \text{ M}^{-1}\). If the same procedure was used for binding at 25°, then with \(n_1 = 1\), \(K_1 = 6.19 \pm 0.35 \times 10^8 \text{ M}^{-1}\), \(n_2 = 0.44 \pm 0.22\), and \(K_2 = 0.73 \pm 0.38 \times 10^8 \text{ M}^{-1}\). These results are in relatively good agreement with the values obtained when no restrictions were made in the nonlinear regression analysis.

Thermodynamic Values for Binding of Thyroid Hormones—As shown in Fig. 6, a plot of log \(K\) or log \(K_1\) against \(1/T\) is linear for thyroxine and close to linear for triiodothyronine, indicating that \(\Delta H^\circ\) is independent of temperature. The thermodynamic values for the interaction of the thyroid hormones with thyroxine-binding globulin in the range 5°-37° are given in Table I. The \(\Delta H^\circ\) values in Table I were estimated graphically from the slopes of the lines in Fig. 6.

Effect of pH on Binding—To determine the effect of pH on binding, measurement was made of \(\psi/A\) values at a fixed molar ratio of thyroid hormone to thyroxine-binding globulin. At the low molar ratio of triiodothyronine to protein used in the pH study (1:10), the observed \(\psi/A\) values are directly related to \(K_1\), since the secondary binding site is estimated to make a contribution of about 10% to the overall binding affinity. In Fig. 7, \(\psi/A\) is plotted as a function of pH for the interaction of thyroxine and triiodothyronine with thyroxine-binding globu-
lin at 25°. Both thyroid hormones have the same pH optimum of binding (Fig. 7) in the range of pH 6.8-7.7. These more extensive studies of pH effect were performed in the absence of ovalbumin. Essentially, similar results were obtained when equilibrium dialysis was done in the presence of ovalbumin at pH 6.70, 7.30, 7.75, and 8.35. With ovalbumin present, thyroxine, and triiodothyronine were bound with almost equal affinity at pH 7.30 and 7.75, while binding was reduced by about 20% and 60% at pH 6.70 and 8.35, respectively.

DISCUSSION

Green et al. (3) had previously determined the association constant for the interaction of thyroxine with thyroxine-binding globulin by performing ultrafiltration measurements at a single molar ratio of thyroxine to binding protein. At pH 7.4 in 0.06 M Tris-HCl buffer, they reported association constants of $2.24 \pm 2.1 \times 10^{-18} \text{M}^{-1}$ and $1.68 \pm 0.60 \times 10^{-18} \text{M}^{-1}$ at 25° and 37°, respectively. Woeber and Ingbar (1) obtained an indirect estimate of $1.7 \times 10^{18} \text{M}^{-1}$ for the association constant at pH 7.4 and 37° based on measurements made in serum following removal of thyroxine-binding prealbumin by immunoadsorption. The value, $K = 9 \times 10^{9} \text{M}^{-1}$ for thyroxine binding found here is somewhat less than these previous estimates.

It should be noted that the fluorescence quenching data for the binding of thyroxine to thyroxine-binding globulin (Fig. 3) differ from those reported by Green et al. (3). These authors obtained a limiting value of about 25% quenching of initial fluorescence of their preparation of thyroxine-binding globulin (3) at 0.85 mol of thyroxine bound/mol of protein. In our results (Fig. 3) about 65% maximal quenching of protein fluorescence.

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Temperature</th>
<th>$K \times 10^{-19}$</th>
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<th>$\Delta H^\circ$</th>
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<td>Thyroxine</td>
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<td></td>
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<td>-12.3</td>
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<tr>
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<td>-11.9</td>
<td>+1.4</td>
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</tbody>
</table>

*In 0.06 M potassium phosphate/0.7 mM EDTA containing 1 mg/ml of ovalbumin.

*Value for $K_1$.

![Fig. 5. Effect of temperature on the binding of triiodothyronine to thyroxine-binding globulin at pH 7.4.](image1)

![Fig. 6. Temperature dependence of binding of thyroxine ($T_1$) and triiodothyronine ($T_3$) at pH 7.4 in 0.06 M potassium phosphate/0.7 mM EDTA buffer. Plot of log $K$ as a function of $1/T$.](image2)

![Fig. 7. Effect of pH on the binding of thyroxine ($T_1$) and triiodothyronine ($T_3$) to thyroxine-binding globulin at 25°. These experiments were done in the absence of ovalbumin. The molar ratios of thyroxine and triiodothyronine with relation to thyroxine-binding globulin were 1:14 and 1:10, respectively.](image3)
Binding of Thyroid Hormones to Thyroxine-binding Globulin

was observed at 0.86 mol of thyroxine bound/mol of protein. This difference in quenching behavior may be due to conformational variations in thyroxine-binding globulin, perhaps arising from the purification procedures (3, 4).

The results indicate that the binding behavior of triiodothyronine is more complex than that of thyroxine. It may be noted that the interaction involving the secondary class of triiodothyronine binding sites is apparently sensitive to temperature, the value for n increasing from 1.4 at 5° to about 0.4 at 37°. At present, we have no explanation for the apparent difference in binding characteristics between the two hormones. Factors that may contribute to the difference could be the larger size and greater negative charge on thyroxine (at pH 7.4, see below) compared to triiodothyronine. For example, owing to its larger size, thyroxine might be prevented from fitting into a second site capable of accepting the smaller triiodothyronine molecule. In addition, if this site were to contain or be very near a negative locus on the protein, then repulsion of the more negatively charged thyroxine molecule would be expected to occur to a much greater extent than for the less negatively charged triiodothyronine.

Estimates of the relative binding affinity of triiodothyronine for the binding protein have ranged from 1/3 to 1/6 the affinity exhibited by thyroxine (14, 15). Our binding data indicate that triiodothyronine is bound about 1/3 as tightly as thyroxine to the globulin at pH 7.4 and 37°.

It is interesting that both thyroxine and triiodothyronine have the same pH optimum of binding, in the range 6.8 to 7.7. At the midpoint about pH 7.2-7.4, the phenolic hydroxyl group of thyroxine (pK = 6.73) is about 80% ionized whereas in triiodothyronine this group (pK = 8.45) (7) is almost 90% in the un-ionized form. Evidence for the involvement of the phenolic hydroxyl group in binding to the globulin has been presented previously (14). The pH dependence of binding of the thyroid hormones to thyroxine-binding globulin contrasts with that observed for binding to albumin (8) and prealbumin (16). Both thyroxine and triiodothyronine are bound optimally to these proteins at pH values corresponding to ionization of their respective phenolic groups. The fact that maximum binding of thyroxine and triiodothyronine occurs at the same pH may indicate the presence at the thyroxine binding site in the protein of an amino group (or groups) which ionizes in this pH region. Two such possibilities are, the imidazole ring of histidine (pK = 6.4-7.0 in proteins) (17) and the terminal a-amino group (pK = 7.4 to 7.9 in proteins) (17).

With regard to the thermodynamic values for binding of thyroxine at pH 7.4 (Table 1), it would appear that the relatively high positive entropy change favoring association may be attributed mainly to hydrophobic forces (18, 19). Hydrophobic interactions would be expected to make an important contribution to binding based on the apolar di-phenyl ether structure in thyroxine and the observation that the fluorescent probe 8-anilino-1-naphthalenesulfonic acid competes for the thyroxine-binding site in the protein (3). An ionic component of binding at pH 7.4, involving the anionic phenolate group of thyroxine and positively charged loci in the protein, would also contribute to the positive entropy term (5). A factor that would be expected to make an important contribution to the negative enthalpy change favoring association at pH 7.4 would be hydrogen bond formation (19, 20) between the protein and the a-amino and carboxylate groups of the alanine side chain of thyroxine, as discussed previously (14). The phenolate group of thyroxine may also be involved in hydrophobic bonding, but the presence of the bulky diortho iodine substituents would be expected to inhibit this type of interaction.

In the case of triiodothyronine (Table 1) the expected large positive change in entropy resulting from the influence of hydrophobic forces is not observed and the main contribution to the change in the free energy at pH 7.4 arises from the relatively large negative change in enthalpy. In addition to possible hydrogen bonding involving the alanine side chain of triiodothyronine, hydrogen bond formation with the phenolic hydroxyl group as donor could also contribute to the -ΔH° term, since the single iodine in the 3'-position of the triiodo compound would be less restrictive of hydrogen bond formation than the 3',5'-iodines in thyroxine. It may be noted that in hydrogen bonding as studied in model systems a negative enthalpy change is usually accompanied by a negative change in entropy (20). Consequently, the resultant small positive change in entropy observed on association of triiodothyronine with the primary binding site on thyroxine-binding globulin at pH 7.4 could be explained if hydrogen bonding were to make a contribution to binding such that its negative entropy component effectively diminishes the large positive change in entropy arising from hydrophobic bonding.

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