ELSEVIER

Journal of Chromatography B, 665 (1995) 303–314

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Characterization of thyroxine–albumin binding using high-performance affinity chromatography II. Comparison of the binding of thyroxine, triiodothyronines and related compounds at the warfarin and indole sites of human serum albumin

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First received 26 July 1994; revised manuscript received 16 November 1994; accepted 24 November 1994

Abstract

High-performance affinity chromatography was used to examine the binding of thyroid hormones and related compounds at the warfarin and indole sites of human serum albumin (HSA). This was studied by continuously applying L-triiodothyronine (L-T_3), L-reverse triiodothyronine (L-rT_3) or structural analogs of these compounds to an immobilized HSA column while making injections of site-specific probe molecules (i.e. *R*-warfarin and L-tryptophan). The results were compared with those obtained previously for L-thyroxine (L-T_4). Equilibrium association constants and thermodynamic parameters measured by this approach showed good agreement with previous models reported for L-T_4 and L-T_3 at their high-affinity sites on HSA. This data confirmed that the phenol groups of L-T_4 and L-T_3 played a significant role in the binding of these compounds at the indole site. Work performed at the warfarin site and with other solutes (e.g. L-rT_3) indicated that additional factors, such as interactions through the thyronine backbone or terminal amine and carboxyl groups of these compounds, could also be involved in the binding of thyroid hormones to HSA.

1. Introduction

The binding of drugs and hormones with proteins in blood is an important process in controlling the distribution, excretion and activity of these compounds in the body [1,2]. The thyroid hormones are one class of compounds for which this type of binding is particularly significant. The main hormones produced by the thyroid gland are L-thyroxine (L-T_4) and L-triiodothyronine (L-T_3). The thyroid gland also secretes an inactive but structurally-related compound known as L-reverse triiodothyronine (L-rT_3). Fig. 1 shows the structures of these molecules and related analogs.

The thyroid hormones are important in regulating a number of biological processes, including oxygen consumption, protein synthesis, carbohydrate metabolism, growth and development, and maintenance of body weight [1,2]. Although L-T_4 has generally been considered to be the main thyroid hormone, some work sug-

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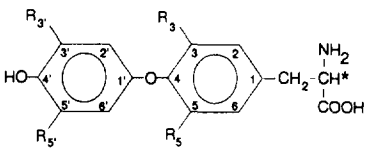
0378-4347/95/$09.50
SSDI 0378-4347(94)00547-8



Compound	R_3	R_5	$R_{3'}$	$R_{5'}$
Thyroxine (T_4)	I	I	I	I
Triiodothyronine (T_3)	I	I	I	H
Reverse Triiodothyronine (rT_3)	I	H	I	I
Diiodothyronine (T_2)	I	I	H	H
Thyronine (T_0)	H	H	H	H

Fig. 1. Structures of the thyroid hormones and related analogs, where the names in parentheses indicate the abbreviations used for these compounds in the text. The terms "I" and "H" represent the presence of iodine or hydrogen, respectively, at the indicated positions. The asterisk (*) shows the chiral center of these molecules.

gests that this may actually act as a prohormone and that L-T_3 is the more biologically significant species [1]. In normal humans, greater than 99.9% of L-T_4 and 99.7% of L-T_3 is protein-bound in blood. This binding is known to occur with three main serum proteins: thyroxine-binding globulin, thyroxine-binding prealbumin, and human serum albumin (HSA) [1,2].

Of these various binding processes, the interactions of thyroid hormones with HSA are the least understood. Many compounds which bind with HSA are believed to interact at a series of relatively well-defined regions on the protein. The two most important of these regions are the warfarin-azapropazone site and the indole-benzodiazepine site [3–5]. In recent crystallographic studies, the locations of these regions have been identified as being in the IIA and IIIA subdomains of HSA, respectively [6]. As their names suggest, these sites are characterized by their ability to bind certain solutes, such as warfarin or indole-containing compounds. Together, these two regions are thought to be involved in the majority of HSA–solute interactions [3,5,7].

The interaction of thyroid hormones with HSA is generally thought to occur at one or two strong binding sites, plus several additional weaker binding regions [2]. In previous work we have used immobilized HSA columns and high-performance affinity chromatography (HPAC) to examine the binding of L-T_4 at both the warfarin and indole sites of HSA. From the results obtained, it was proposed that these sites were the two main binding regions for L-T_4 on this protein [8]. Like L-T_4, the hormone L-T_3 is also believed to have one or two strong binding sites on HSA. The equilibrium association constants for L-T_3 at these sites are generally thought to be in the range 10^4–10^5 M^{-1} [2,9]. However, the exact values of these constants and location of the corresponding binding sites are still unclear. Even less data is available regarding the interactions of L-rT_3 with HSA.

This work will use HPAC and the technique of zonal elution to examine the binding of L-T_3 and L-rT_3 at the warfarin and indole sites of HSA. This will be performed by continuously applying known concentrations of L-T_3 or L-rT_3 to an immobilized HSA column while small injections are made of a site-specific probe molecule (i.e. *R*-warfarin or L-tryptophan). From the results, the equilibrium association constants for L-T_3 and L-rT_3 at each site will be determined. The changes in free energy associated with these interactions will also be measured by repeating the zonal elution experiments at several temperatures. The data obtained for L-T_3 and L-rT_3 will then be compared with the results of similar experiments performed with L-T_4 or several thyroid hormone analogs. From these studies, information will be obtained that can be used to provide a better understanding of the mechanisms involved in the binding of thyroid hormones to HSA.

2. Theory

The use of affinity chromatography as a tool for measuring the equilibrium constants of biological systems is an approach that is now well-established [10,11]. The method of zonal elution was used in this present study to examine the competition of thyroid hormones and related compounds with probe molecules injected onto

an immobilized HSA column. In this technique, a known concentration of the solute of interest (S) is continuously applied to a column containing an immobilized ligand (L) while small injections of a site-specific probe (P) are made [8]. If S and P compete at a single site on L and P binds to no other sites on the matrix, then the following relationship can be derived to describe the retention of P at various concentrations of the applied solute [8,10]:

$$\frac{1}{k'_P} = \frac{K_S V_m [S]}{K_P m_L} + \frac{V_m}{K_P m_L} \quad (1)$$

where K_S and K_P are the equilibrium association constants for the binding of S and P to L at the site of competition, $[S]$ is the concentration of solute applied to the column, V_m is the void volume of the column, and m_L is the number of moles of common binding sites. The term k'_P is the capacity factor for solute P, as given by the expression $k'_P = t_{r,p}/(t_m - 1)$, where $t_{r,p}$ is the measured retention time of the probe and t_m is the void time of the column.

For a system with single-site competition, Eq. (1) predicts that a plot of $1/k'_P$ vs. $[S]$ will yield a linear relationship with a slope of $(K_S V_m / K_P m_L)$ and an intercept of $(V_m / K_P m_L)$. By calculating the ratio of the slope to the intercept for such a plot, the value of K_S can be directly obtained. One advantage of this approach is that it provides an association constant for S that reflects binding only at the site of competition with P. This is useful in studying a system with multi-site interactions, such as the binding of thyroid hormones to HSA, because it allows individual sites to be monitored through the injection of appropriate probe molecules [8].

One assumption made in the use of Eq. (1) is that the amount of injected probe is small with respect to the total amount of active ligand in the column. In other words, it is assumed that linear elution conditions are present, or that the retention time measured for the probe is independent of the probe's initial sample concentration. This assumption can be tested by varying the concentration of the probe and by determining whether or not there is a shift in the resulting retention time. If a change is observed, then the probe concentration can be decreased until such changes are no longer present.

Another assumption made in Eq. (1) is that the mean position of the probe's peak (i.e. its capacity factor or central moment) represents the establishment of a local equilibrium between the probe and the ligand as the sample passes through the column. In order for this to be true, the kinetics of binding and dissociation between these two species must be fairly rapid compared to the overall column residence time of the probe. This can be tested by performing zonal elution studies at several flow-rates and by determining whether or not the resulting capacity factors show any flow-rate (or column residence time) dependence.

If zonal elution is used to determine equilibrium association constants at several temperatures, the changes in the free energy of a solute–ligand system can be obtained as follows:

$$\ln K = -\Delta H/\mathrm{RT} + \Delta S/R \quad (2)$$

$$\Delta G = -\mathrm{RT} \ln K \quad (3)$$

where K is the equilibrium association constant measured at a given absolute temperature (T) and R is the ideal gas law constant. The term ΔG is the change in total free energy of the reaction, ΔH is the change in free energy due to enthalpy, and ΔS is the change in entropy. Eq. (2) predicts that a plot of $\ln K$ vs. $1/\mathrm{T}$ will yield a linear relationship with a slope of $-\Delta H/R$ and an intercept of $\Delta S/R$. This provides the values of ΔH and ΔS for the reaction. By using Eq. (3) and the values of K and T, ΔG for the reaction can also be obtained [12].

3. Experimental

3.1. Reagents

HSA (Cohn fraction V, 99% globulin-free), L-tryptophan, and thyroid hormones or related analogs were from the Sigma (St. Louis, MO, USA). *R*(+)-Warfarin was generously provided by DuPont Pharmaceuticals (Wilmington, DE,

USA). The HSA and diol-bonded silica supports were prepared using Nucleosil Si-1000 (7 μm particle diameter, 1000 Å pore size) from Alltech (Deerfield, IL, USA). Other chemicals and biochemicals were of the purest grades available. All solutions were prepared with water purified by a Nanopure water system (Barnstead, Dubuque, IA, USA).

3.2. Apparatus

The chromatographic system consisted of one CM3000 isocratic pump and one SM3100 UV-Vis variable-wavelength absorbance detector from LDC/Milton Roy (Riviera Beach, FL, USA). Samples were applied using a Rheodyne 7010 valve (Cotati, CA, USA) equipped with an injection port and a PhaseSep event marker (Phase Separations, Queensferry, UK). Data were collected using a ThermoChrom Model II interface and software from LDC/Milton Roy. Chromatograms were processed by programs written in Microsoft QuickBASIC (Redmond, WA, USA) using double-precision logic. Columns and mobile phases were temperature-controlled using an Isotemp 9100 circulating water bath (Fisher Scientific, Pittsburgh, PA, USA). Columns were prepared using an HPLC column slurry packer from Alltech.

3.3. Methods

The HSA and diol-bonded silica supports used in this work were prepared as described previously [8,13]. The protein content of the immobilized HSA support was 204 ± 1 (1 S.D.) nmol per gram of silica, as determined in duplicate by a bicinchoninic acid protein assay [14]. The HSA and diol-bonded silica supports were downward slurry-packed at 28 MPa (4000 psi) into two separate 45 × 4.1 mm I.D. columns of a previously published design [15]. Both columns were enclosed in water jackets for temperature control. All studies, except those examining the temperature dependence of thyroid hormone–HSA binding, were performed at 37 ± 0.2°C. All mobile phases and packing solvents used in this work were prepared using 0.067 *M* potassium phosphate buffer (pH 7.4). Prior to use, the mobile phases were filtered through a 0.45-μm cellulose acetate filter and degassed under vacuum for 10 min.

Elution of *R*-warfarin and L-tryptophan was detected by either monitoring at a wavelength near the absorbance maximum for each probe (i.e. 290 nm for L-tryptophan or 310 nm for *R*-warfarin) [8] or by using an intermediate detection wavelength of 300 nm. Monitoring at 300 nm was particularly convenient since both probes had significant absorbance at this wavelength and these conditions avoided the need to change the detector settings between probe injections.

Zonal elution studies were performed on the HSA column by applying pH 7.4 buffer containing 0–27 μM of the desired test solute while making 20-μl injections of *R*-warfarin or L-tryptophan onto the column. All thyroid hormone and analog solutions used in this study were made from a stock that was initially prepared in 0.067 *M* phosphate buffer (pH 11.0) and slowly adjusted to pH 7.4 by the addition of 0.067 *M* phosphate buffer (pH 6.0) at room temperature. This solution was then diluted to the desired concentration by adding 0.067 *M* phosphate buffer (pH 7.4). The thyronine (T_0), diiodothyronine (T_2), rT_3 and T_4 solutions that were prepared in this manner were found to be stable for one week when stored at room temperature. The T_3 solutions made in this fashion were stable for up to three days under the same conditions.

The warfarin and tryptophan samples were prepared by dissolving 2.5–6.5 μM *R*-warfarin or 1.5–4.0 μM L-tryptophan in the mobile phase to be used in the experiment. No apparent shifts in the capacity factors were noted when varying the probe concentrations over these ranges, in agreement with the results of previous studies [16]. The flow-rates used for probe injection ranged from 0.1 to 0.5 ml/min, with no significant changes in the capacity factor being noted for either compound under this range of conditions.

The retention times of the warfarin and tryptophan peaks were calculated from their central moments [17]. The column void volume was

determined by making injections of warfarin or tryptophan onto the column containing only diol-bonded silica. All elution times were corrected for the extra-column volume of the chromatographic system, as determined by making injections of *R*-warfarin or L-tryptophan when no column was present.

4. Results and discussion

4.1. Column characterization

Previous studies have examined the binding properties of the HSA column used in this work [14] and of similar supports prepared by the same immobilization method [8,16]. Two test solutes which have been employed in these studies are *R*-warfarin [8,14] and L-tryptophan [8,16]. These compounds are of interest since they are believed to have 1:1 interactions at the warfarin and indole sites of HSA, respectively [18–20]. With both solutes, it has been found that the association constants measured on such HSA columns are generally within 1–15% of the values reported for HSA in solution [8,14]. In addition, the binding of *R*-warfarin and L-tryptophan to the immobilized HSA fits a model in which these compounds are interacting at single, but distinct sites on the protein [8,14,16]. This is in agreement with earlier solution-phase experiments [18–20] and crystallographic studies [6]. These data indicate that the support used in this study can serve as a model for examining the interactions of compounds at the warfarin and indole sites of HSA [8,14]. Similar conclusions have been reached in other studies that have utilized immobilized HSA columns as tools for the study of drug–protein interactions [21,22].

The HSA column used in this work was operated over the course of nine months and 500 sample injections. During this time, the capacity factors for *R*-warfarin and L-tryptophan, as measured in the absence of a competing agent, decreased by about 60%. This was directly related to a decrease in the column's binding capacity, as determined by repeated frontal analysis experiments [14]. However, the association constants measured on the HSA column varied by less than 5–10% over the same time period [14]. Similar reproducibility in equilibrium constant measurements performed by HPAC has been noted in previous work [8]. This is due to the use of intercept and slope ratios in expressions like Eq. (1), which helps to correct for any long-term variations in the binding capacity of the column [8,14].

4.2. Equilibrium constants for thyroid hormone binding to HSA

After it had been confirmed that the HSA column could be used to model interactions at the warfarin and indole sites of HSA, zonal elution experiments were performed to examine the binding of thyroid hormones and related compounds at these sites on the column. An example of one such study is shown in Fig. 2. In this experiment, *R*-warfarin was injected as the probe while solutions of L-rT_3 were continuously applied to the column. As the concentration of L-rT_3 was increased, a decrease was observed in the elution time of the *R*-warfarin peak. This change in retention indicated that some type of competition was taking place between *R*-war-

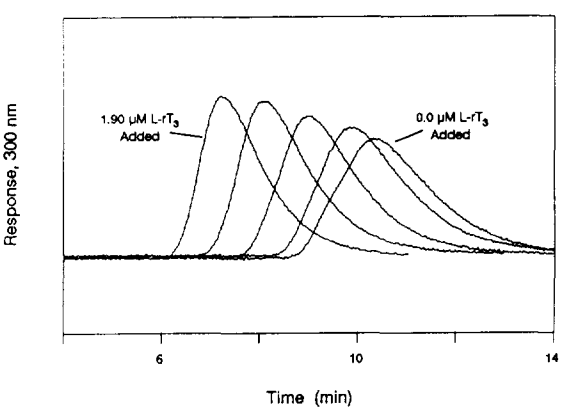


Fig. 2. Zonal elution studies using injections of *R*-warfarin onto an immobilized HSA column in the presence of buffers containing various concentrations of L-rT_3 at 37°C. The concentrations of L-rT_3 used in these studies (from left to right) were 1.90, 0.97, 0.49, 0.24 and 0 μM, respectively. The samples consisted of 6.5 μM *R*-warfarin dissolved in the corresponding mobile phase. These samples were injected in 20-μl aliquots onto the column at a flow-rate of 0.5 ml/min.

farin and L-rT$_3$ in the column. Similar behavior was seen for most of the other solutes tested when either *R*-warfarin or L-tryptophan was injected as the probe molecule.

A more detailed examination of the data in Fig. 2, and related studies, was made by plotting the results according to Eq. (1). Fig. 3 shows the results obtained for L-rT$_3$. Both this compound and L-T$_3$ gave linear behavior for such graphs, with correlation coefficients in the range 0.9976–0.9999 (mean, 0.9814 for $n = 4$) over the five to seven concentrations studied. According to Eq. (1), this behavior indicated that there was single-site competition between these solutes and the injected probe compounds [8].

For both L-rT$_3$ and L-T$_3$, there was good agreement between the capacity factors observed for the probe molecules in the absence of the test solutes and the capacity factors predicted from the intercepts of best-fit lines. In each case a difference of less than 4% in these values was observed, will all experimental values falling within ± 2 S.D. of the predicted intercepts. This agreement indicates that the equilibrium association constants for the probe molecules, and thus

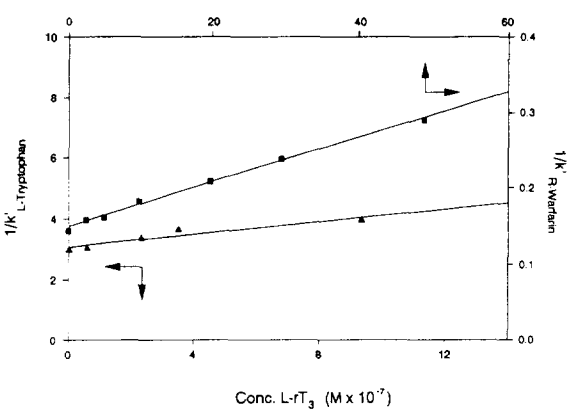


Fig. 3. Results of zonal elution studies performed at 37°C for *R*-warfarin (■) or L-tryptophan (▲) injected onto an immobilized HSA column in the presence of L-rT$_3$. These data were plotted according to Eq. (1) in the text. The best-fit line for the warfarin site data is $y = x\,[2.96(\pm 0.09) \times 10^4\ M^{-1}] + [0.149 \pm 0.002]$, with a correlation coefficient of 0.9976 ($n = 7$). The best-fit line for the indole site data is $y = x\,[1.03(\pm 0.21) \times 10^6\ M^{-1}] + [3.1 \pm 0.1]$, with a correlation coefficient of 0.9424 ($n = 5$).

their binding sites, were not affected by allosteric interactions over the concentrations of L-T$_3$ and L-rT$_3$ that were used in this study [8]. These results support a model in which there is direct competition between L-T$_3$ and L-rT$_3$ with the probe molecules at the warfarin and indole sites of HSA. This same type of behavior has been noted for the binding of L-T$_4$ at these sites [8].

By using Eq. (1) and the best-fit parameters for L-T$_3$ and L-rT$_3$, the equilibrium association constants could be determined for these compounds at the warfarin and indole sites of HSA. The results are given in Table 1. The precision of the association constants measured for these compounds were between ± 0.6 and 18% (± 1 R.S.D.). In previous studies, it has been found that L-T$_4$ shows strong binding at both the warfarin and indole sites of HSA [8]. The results obtained here with L-T$_3$ and L-rT$_3$ indicate that these compounds also have relatively strong binding for these regions. As shown in Table 1, the association constants for L-T$_3$ at the warfarin and indole sites are both approximately $2 \times 10^4\ M^{-1}$ at 37°C. For L-rT$_3$, the analogous association constants are ca. $2–3 \times 10^5\ M^{-1}$. The values measured previously for L-T$_4$ are similar to those seen for L-rT$_3$.

The results of this work are in good agreement with those of earlier studies that have examined the binding of L-T$_3$ with HSA. For example, the

Table 1
Association constants for the binding of thyroid hormones and related analogs to HSA at 37°C[a]

Compound	Association constant, K ($M^{-1} \times 10^5$)	
	Warfarin site	Indole site
L-T$_4$[b]	1.4 ± 0.1	5.7 ± 0.8
L-T$_3$	0.170 ± 0.001	0.25 ± 0.02
L-rT$_3$	1.99 ± 0.07	3.3 ± 0.6
L-T$_2$	0.63 ± 0.03	1.16 ± 0.06
L-T$_0$	0.18 ± 0.02	–
D-T$_4$	5.5 ± 0.9	29 ± 2
D-T$_3$	1.45 ± 0.06	1.2 ± 0.2

[a] The value following each association constant represents a range of ± 1 S.D. All association constants were measured at pH 7.4 in 0.067 *M* phosphate buffer.
[b] The results given for L-T$_4$ were obtained from Ref. [8].

association constants determined for L-T_3 at the warfarin and indole sites fall within the range 10^4–10^5 M^{-1} reported at the high-affinity sites of HSA for this compound [2]. These results also agree qualitatively with the comparison of HSA binding to L-T_4 and L-T_3 that was made by Steiner et al. [24]. A similar comparison of the L-rT_3 results with those reported in the literature was not possible since there are no known reports of the association constants for this compound with HSA.

It has been suggested in earlier work that the indole and warfarin sites of HSA are the high-affinity binding regions for L-T_4 on this protein. This was concluded by comparing the association constants and thermodynamic parameters measured at these sites with literature values reported for the high-affinity binding regions of L-T_4 on HSA [8]. The importance of the indole site in L-T_4 interactions has also been demonstrated by Tritsch and Tritsch [25]. The data presented here indicate that this site and the warfarin binding region may also be the main sites for L-T_3 on HSA. However, it should be noted that these results do not rule out the possible existence of other high-affinity regions because only the warfarin and indole sites were sampled in these studies.

4.3. Temperature studies

The role of temperature in the binding of L-T_3 and L-rT_3 to HSA was examined by performing several zonal elution experiments between 4 and 45°C. At each temperature studied, plots made according to Eq. (1) produced the same type of linear behavior as seen in Fig. 3. The correlation coefficients of these plots ranged from 0.9116 to 0.9997 (mean, 0.9733 for $n = 16$) for the four to seven concentrations of L-T_3 and L-rT_3 studied. In all of these graphs, the intercepts of the best-fit lines showed good agreement with the capacity factors measured experimentally in the absence of any competing agent. In a comparison of these latter values, it was found that 94% of the experimental results were within ± 2 S.D. of the predicted intercepts and the other 6% were within ± 3 S.D. As noted earlier, this agreement indicates that there was direct competition between *R*-warfarin and L-tryptophan with L-rT_3 and L-T_3. This also indicates that the conditions used in this work did not produce any detectable allosteric effects in the binding of the probe molecules due to the interactions of L-rT_3 and L-T_3 at regions on HSA other than the warfarin and indole sites [8].

From the association constants measured in the temperature studies, plots of ln K versus 1/T were made according to Eq. (2). Some examples of these plots are shown in Fig. 4. All graphs made in this fashion for L-T_3 and L-rT_3 showed a linear behavior, with correlation coefficients ranging from 0.7252 to 0.8814 (mean, 0.8222 for $n = 4$) over the four temperatures studied.

In these studies, it was found that the association constants for L-T_3 and L-rT_3 changed slightly with temperature. In the case of L-T_3, the association constants decreased by 50% and 39% for the warfarin and indole sites, respectively, in going from 4 to 45°C. For L-rT_3, the association constants for these sites decreased by 33% to 43% over the same temperature range. This temperature dependence is similar to that observed in the binding of L-T_4 with HSA [8,23].

On the basis of circular dichroism [26], fluores-

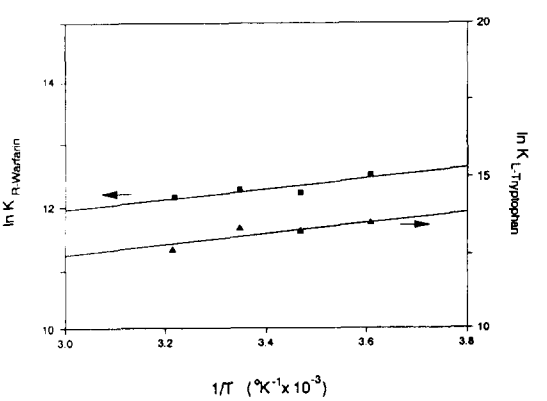


Fig. 4. Effect of temperature on the binding of L-rT_3 at the warfarin (■) and indole sites (▲) of an immobilized HSA column. These data were plotted according to Eq. (2) in the text. The best-fit line for the warfarin site data is $y = x\,[0.8(\pm 0.3) \times 10^3] + [9.4 \pm 0.1]$, with a correlation coefficient of 0.8802 ($n = 4$). The best-fit line for the indole site data is $y = x\,[1.8(\pm 0.7) \times 10^3] + [6.9 \pm 0.2]$, with a correlation coefficient of 0.8814 ($n = 4$).

cence quenching [24], and equilibrium dialysis experiments [23,27], the binding of L-T_4 at its major site(s) on HSA has previously been described by using a model in which the C1′–C6′ ring of L-T_4 is placed deep into the binding region. The amine and carboxyl groups of L-T_4 are viewed as being located at or near the surface of the binding site, placing both aromatic rings and all four iodines of L-T_4 within the binding pocket [26]. In this model, interactions between the ionized phenol group on L-T_4 and the protonated amino acid residues on HSA are thought to be the main forces favoring formation of the L-T_4–HSA complex [23,27]. Other forces which may contribute to this binding include hydrophobic interactions, hydrogen bonding, and dipole interactions [26,27]. However, the relative importance of these forces versus phenol group ionic interactions has not yet been determined [26,27].

The mechanisms and forces involved in the binding of L-T_4, L-T_3 and L-rT_3 with HSA were examined more closely in this study by determining the changes in free energy that occurred during the interactions of these compounds at the warfarin and indole sites. By applying Eqs. (2) and (3) to plots like those in Fig. 4, it was possible to determine ΔG, ΔH and ΔS for the binding of L-T_3 and L-rT_3 at the warfarin and indole sites of HSA. The results obtained are given in Table 2. Previous values determined for L-T_4 [8] are also provided for comparison. The total change in free energy (ΔG) determined for the binding of L-T_3 at the warfarin and indole sites ranged from −6 to −6.2 kcal/mol at 37°C. At the same temperature, the value of ΔG for L-rT_3 was found to range from −7.5 to −7.8 kcal/mol.

For each of these compounds, a significant portion of the energy gained during HSA binding is due to the entropy term ($-T\Delta S$). This change in entropy is related to the release of water during solute–protein binding [23]. As shown in Table 2, the entropy terms for L-T_3 and L-rT_3 make up about 50–80% of the total change in free energy measured for HSA binding at 37°C. At the same temperature, this term makes up 50–85% of the total change in free energy observed for L-T_4 [8]. This indicates that the release of solvent, or gain in entropy, that occurred during these binding processes was important in determining the interaction strength of these compounds at the warfarin and indole sites. A similar conclusion has been reached in studies by Tabachnick, which examined only L-T_4/HSA binding [23].

A comparison of the results for L-T_4, L-rT_3 and L-T_3 in Table 2 shows that there is a wide

Table 2
Thermodynamics of HSA binding for L-T_4, L-T_3 and L-rT_3 at the warfarin and indole sites[a]

Compound/region	ΔG at 37°C (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/mol°K)	$-T\Delta S$ at 37°C (kcal/mol)
L-T_4[b]				
Warfarin site	−7.3 ± 1.1	−3.7 ± 0.8	12 ± 3	−3.6 ± 1.0
Indole site	−8.2 ± 0.6	−1.2 ± 0.4	23 ± 1	−7.0 ± 0.3
L-T_3				
Warfarin site	−6.0 ± 0.3	−3.0 ± 2.0	10 ± 6	−3.0 ± 1.9
Indole site	−6.2 ± 0.1	−2.1 ± 1.1	13 ± 4	−4.1 ± 1.2
L-rT_3				
Warfarin site	−7.5 ± 0.1	−1.7 ± 0.6	19 ± 0.2	−5.8 ± 0.1
Indole site	−7.8 ± 0.1	−3.6 ± 0.4	14 ± 0.4	−4.2 ± 0.1

[a] The numbers following each value represent a range of ± 1 S.D. The ΔG values for L-T_3 and L-rT_3 were calculated using Eq. (3) and the data in Table 1.
[b] The results given for L-T_4 were obtained from Ref. [8].

variation in the changes in entropy noted for these compounds during their binding to HSA. At the indole site of HSA, the values observed for $-T\Delta S$ ranged from -4.1 to -7.0 kcal/mol. L-T_4 had the biggest change in entropy at this site, followed by L-T_3 and L-rT_3, which gave similar values. At the warfarin site, the values measured for $-T\Delta S$ ranged from -3.0 to -5.8 kcal/mol. The order of the test compounds at this region, as ranked according to their changes in entropy (i.e. L-rT_3 > L-T_4 > L-T_3), was significantly different from that seen at the indole site.

The greater change in entropy noted for L-T_4 versus L-T_3 at the indole region is consistent with the previous model used to describe the binding of L-T_4 at its highest affinity site on HSA. Recall that this model considers interactions between the ionized phenol group on L-T_4 and positively-charged amino acid residues on HSA to be an important factor in stabilizing the final L-T_4–HSA complex [23,27]. It is known that the phenol group of L-T_4 ($pK_a = 6.73$) is more acidic than the phenol group of L-T_3 ($pK_a = 8.45$) [28]. In free solution, this means that about 82% of the L-T_4 phenol groups are ionized at pH 7.4 while only 8% of the phenol groups on L-T_3 are ionized under the same conditions [27]. This difference can be significant since it is known that coulombic interactions in an aqueous environment generally involve a large change in entropy due to the significant amount of desolvation that is required to form the final ion pair [29].

Although the data seen for L-T_4 and L-T_3 at the indole site agree with a model based on phenol group interactions, the results obtained for L-rT_3 at the same region indicates that other factors are also important in determining the overall binding strength of these compounds. For example, in previous work it has been reported that 3,5-diiodotyrosine compounds, such as L-rT_3, have pK_as for their phenol groups that are similar to that of L-T_4 [28]. However, the change in entropy measured for L-rT_3 at the indole site was much less than that noted for L-T_4 and was essentially the same as that seen for L-T_3 (see Table 2).

Other researchers have suggested that forces besides those involving the phenol groups of L-T_4 and L-T_3 might be involved in the binding of these compounds to their major sites on HSA [24,27]. For example, at the indole site of HSA, simple displacement of solvent by the additional iodine on L-T_4 may have contributed to the greater change in entropy seen for this compound versus L-rT_3 and L-T_3. The similarity in $-T\Delta S$ values noted for L-rT_3 and L-T_3 at the indole site may indicate an alteration in phenol group acidity for these compounds upon their binding to HSA [24] or the presence of interactions involving other portions of these molecules [27].

The changes in entropy measured at the warfarin site for L-T_4, L-T_3 and L-rT_3 also suggest the presence of interactions which involve regions of these molecules other than their ionized phenol groups. In this case, L-rT_3 had the largest change in entropy, with L-T_4 and L-T_3 having similar, lower values for $-T\Delta S$. This is probably associated with the difference in fit that is created by the presence of the C5 iodine in L-T_4 and L-T_3.

The changes in energy due to enthalpy (ΔH) measured during the binding of L-T_4, L-rT_3 and L-T_3 to HSA were all in the range of -1.2 to -3.7 kcal/mol. These values fall within the wide range of energies that might be expected for protein–solute binding due to hydrophobic interactions, electrostatic interactions and/or hydrogen bonding [29,30]. Because any one of these interactions, or any number of interaction combinations, could account for these enthalpy changes, no details on the specific mechanisms involved in the solute–HSA interactions could be determined directly from these values.

4.4. Binding of thyroid hormone analogs to HSA

More data on the mechanisms of interaction for L-T_4, L-T_3 and L-rT_3 with HSA were obtained by comparing the binding strengths of these compounds with those measured for various structural analogs (see list given in Table 1). The association constants of these analogs were mea-

sured as described for L-T_3 and L-rT_3, by using zonal elution studies with injections of *R*-warfarin or L-tryptophan as the probe compounds. The association constants determined from these experiments are summarized in Table 1.

Plots made according to Eq. (1) gave the same type of linear behavior as noted in Fig. 3 for all but one of the analogs tested. These plots had positive slopes with correlation coefficients in the range of 0.9286 to 0.9934 (mean, 0.9677 for $n = 7$) over the four to six concentrations examined. The intercepts obtained from the capacity factors measured for the probes were all within ± 2 to 3 S.D. of the best-fit intercept values. The only graph in these studies that gave a different type of behavior was that seen for L-thyronine (L-T_0) when it was used with injections of L-tryptophan. In this latter case, a plot made according to Eq. (1) resulted in a negative slope, indicating that some type of cooperative binding was taking place between L-tryptophan and L-T_0 in the HSA column.

The importance of the amine and carboxylic acid groups on thyroid hormones in determining HSA binding was examined by comparing the binding strengths measured for the D- and L-enantiomers of T_3 and T_4. The association constants for the D-enantiomers were four to nine times larger than those measured for the corresponding L-enantiomers (see Table 1). The difference in total free energy ($\Delta\Delta G$) measured at 37°C for the D- versus L-forms of T_3 and T_4 at the warfarin site ranged from −0.8 to −1.3 kcal/mol. At the indole site, this energy difference was −0.9 to −1.0 kcal/mol. These results indicate that there may be some interactions involving the amine and carboxyl groups of thyroid hormones at both of the HSA regions that were sampled. However, these results also show that such interactions are strongest for the D-enantiomers of T_3 and T_4.

Another factor examined in this work was the effect of the position and number of iodines on the binding of thyroid hormones to HSA. The potential importance of iodines located at the C3′ and C5′ positions was discussed earlier. The role of iodines at the C3 and C5 positions was studied by comparing the association constants measured for L-T_2 with those seen for L-T_4, L-rT_3, L-T_3 and L-T_0 (see Table 1). The value of ΔG for L-T_2 interactions at the indole site differs by +0.6 to +1.0 kcal/mol versus the binding energies determined for L-T_4 and L-rT_3, respectively. At the warfarin site, the value of ΔG for L-T_2 differs by +0.5 to +0.7 kcal/mol versus the values for these same reference compounds. These differences in energy may be related to the greater degree of phenol group ionization or solvent displacement that would be expected to occur during the binding of the 3′,5′-diiodinated compounds to HSA.

Note in Table 1 that L-T_2 has stronger binding than L-T_0 at the warfarin site of HSA. This demonstrates the importance of the C3 and/or C5 iodines in the stabilization of thyroid hormone binding at this region. Table 1 also indicates that the interactions of L-T_2 at the warfarin and indole sites are stronger than those seen for L-T_3. It is known that the phenol group of L-T_2 ($pK_a = 9.29$) is less ionized at physiological pH than the phenol group of L-T_3 ($pK_a = 8.45$) [28]. Thus, the stronger binding of L-T_2 is probably not due to differences in the ionic interactions which occur through the phenol group. One possible explanation is that the C3′ iodine of L-T_3 creates steric hindrance and/or twisting of this compound as it fits into the HSA binding pockets. If this is true, then the same phenomenon would also be likely to occur with L-rT_3 and L-T_4.

The last item examined in this work was the role played by the thyronine backbone in determining the binding of thyroid hormones to HSA. Experiments with L-T_0 were used to examine these types of interactions. As already mentioned, the cooperative binding produced between L-T_0 and L-tryptophan made it difficult to use this combination of compounds to study L-T_0 binding at the indole site. However, it was possible to examine the interactions of L-T_0 at the warfarin site. It is interesting to note that the association constant measured for L-T_0 at the warfarin site is statistically identical to that seen for L-T_3. This suggests that interactions via the amine and carboxyl end or aromatic backbone of L-T_3 play a major role in the binding of this compound at the warfarin site. These same interactions would also be expected to play at

least some part in the retention of L-T_4 and L-rT_3 by this region of HSA.

5. Conclusions

This study used high-performance affinity chromatography to investigate the binding mechanisms of thyroid hormones and related analogs to HSA. By using an immobilized HSA column and *R*-warfarin or L-tryptophan as site-specific probes, it was possible to selectively measure the equilibrium association constants for L-T_3, L-rT_3 and other compounds at the warfarin and indole sites of HSA. The changes in entropy, enthalpy and total free energy that were produced during the binding of L-T_3 and L-rT_3 were also examined. These data were then compared with those obtained earlier in similar experiments performed with L-T_4 [8]. Good agreement was seen between the results of this work and previous measurements reported for the binding of thyroid hormones at their high-affinity sites on HSA. These results suggest that the warfarin and indole sites of HSA are the locations of these high-affinity regions.

The mechanisms of thyroid hormone interactions at the warfarin and indole sites were examined by comparing the thermodynamic parameters for these processes and by studying the retention of these sites for various thyroid hormone analogs. The thermodynamic studies indicated that a significant portion of the energy gained from the binding of thyroid hormones to the warfarin and indole regions was due to an associated increase in the entropy of the system. The changes in entropy that were noted for L-T_4 versus L-T_3 at the indole site were consistent with a previous model that has been used to describe the binding of L-T_4 at its high-affinity site on HSA based on ionized phenol group interactions [23,27]. However, the change in entropy noted for L-rT_3 at the indole site, and that seen for all compounds at the warfarin site, indicated that other forces also played a role in determining the overall binding strength of these compounds. Experiments with various thyroid hormone analogs demonstrated that the number and position of iodines in these compounds can have a large effect on the binding of these molecules at the warfarin and indole sites. Ways in which the iodine substituents might affect this binding include the changes that they may induce in phenol group acidity, solvent displacement or steric hindrance of the hormone during its interactions with HSA. Work performed with L-thyronine at the warfarin site indicated that interactions between HSA and the carbon backbone of thyroid hormones may also affect these binding processes. In addition, studies using the D- and L-enantiomers of T_4 and T_3 demonstrated that some binding can take place via the terminal amine or carboxyl groups of thyroid hormones, with the D-enantiomers showing the strongest interactions at both the warfarin and indole sites.

In this and previous studies, high-performance affinity chromatography has been demonstrated to be a powerful tool for the study of biomolecular interactions. Advantages of this approach in equilibrium constant measurements include its speed, reproducibility, and precision [8,10,14,16]. In the particular example given here, it was necessary to immobilize the ligand of interest (i.e. HSA) prior to the experiment; however, the time and effort required for this was minimal when compared to the fact that this support could be used over the course of several hundred injection cycles [8,16].

The ability to use high-performance affinity columns with site-specific probes is convenient since it allows detailed information to be obtained on individual binding regions in systems with multi-site interactions [8]. As shown in this work, the information that can be provided by this method includes not only the equilibrium constants for specific sites, but also information regarding the mechanisms of solute–ligand interactions at these regions. These properties make HPAC an attractive approach for the study of protein binding to hormones, drugs and other compounds.

Acknowledgement

This work was supported by the National Institutes of Health under grant No. GM44931.

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