Binding Characteristics of a Thyroid Hormone Metabolite, 3,3'-Diiodo-L-thyronine, to Bovine Serum Albumin as Measured by Fluorescence

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The interaction between a thyroid hormone metabolite, 3,3'-diiodo-L-thyronine(3,3'-T₂) and bovine serum albumin (BSA) was investigated by the fluorescence method. The apparent binding constants and thermodynamic parameters were obtained assuming the equivalence and independence of the binding sites on the BSA molecule. The maximum binding was attained at near pH 8.5 and the apparent binding constant at pH 8.5 was $2.7 (0.2) \times 10^5 \text{ M}^{-1}$. The standard free energy change, enthalpy change and entropy change were $-7.36 (0.02) \text{ kcal mol}^{-1}$, $-7.75 \text{ kcal mol}^{-1}$ and -1.51 e.u. , respectively.

Keywords thyroid hormone metabolite; thyroid hormone; hormone binding; 3,3'-diiodo-L-thyronine; bovine serum albumin; serum albumin binding; binding constant; thermodynamic parameter; fluorescence

Introduction

Thyroid hormone, 3,5,3',5'-tetraiodo-L-thyronine (T₄) and 3,5,3'-triiodo-L-thyronine (T₃) are well known as being the hormonogenically active forms.¹⁻³⁾ The binding characteristics of these active hormones with serum albumin have been investigated extensively by fluorescence, equilibrium dialysis and circular dichroism measurements.⁴⁻⁹⁾ Contrasting with these hormonogenically active forms, little is known about the binding properties of the thyroid hormone metabolite, 3,3'-diiodo-L-thyronine (3,3'-T₂). 3,3'-T₂, it is the main deiodinated metabolite of T₃ and 3,3',5'-triiodo-L-thyronine (r-T₃), has an extremely high metabolic clearance rate relative to other iodothyronines, and is contained at about 9–28 ng/dl in serum.⁹⁾ Although the physiological functions of 3,3'-T₂ are not yet clear, it is important to clear its binding characteristics with serum albumin in order to obtain information about the transport of the deiodinated thyroid hormone metabolites through blood serum. In this study, the interaction between 3,3'-T₂ and bovine serum albumin (BSA) was investigated by fluorescence measurements at various pH's and temperatures.

Materials and Methods

BSA (Lot No. 9365) was obtained from Sigma Chemical Co. 3,3'-T₂ (Lot No. 12100) was from Reinstsubstanzen Henning Berlin. Other reagents were of the highest quality available and obtained from Nakarai Chemicals, Ltd.

Fluorescence measurements were performed with a Hitachi 850 spectrofluorometer. The temperature of the sample was controlled by the use of a hollow cell holder through which water from a constant temperature bath regulated within 0.1 °C was circulated and measured directly by using a Takara thermister D641. The fluorescence excitation and emission wavelengths were 280 nm and 340 nm, respectively. In a typical experiment, aliquots (0.5–1.0 μl) of 1 mM 3,3'-T₂ were added to 200 μl of a 1 μM BSA solution in 0.1 M of sodium phosphate buffer in a 0.5 × 0.5 cm² quartz micro cuvette under stirring. The observed relative intensity was corrected for dilution of the albumin. The BSA concentration was determined spectrophotometrically using, $E_{1\%}^{1\text{cm}} = 6.54$ at 280 nm¹⁰⁾ and the molecular weight of 66300¹¹⁾ in 0.1 M sodium phosphate buffer, pH 7.5. The apparent binding constants and the number of binding sites were evaluated from the fluorescence quenching titration of BSA with 3,3'-T₂ according to Attallah and Lata¹²⁾ assuming the equivalence and independence of the binding sites.

$$K = \frac{Q_f}{(1 - Q_f)(T_i - nQ_fP_i)}$$

$$Q_f = \frac{f_0 - f_m}{f_0 - f_r}$$

where K is the apparent binding constant, Q_f is the ratio of quenching at a point on the quenching curve to the maximum quenching, T_i is the total concentration of 3,3'-T₂ added, P_i is the total BSA concentration, n is the number of 3,3'-T₂ bound to a BSA molecule, f_0 is the fluorescence intensity of the albumin solution without added 3,3'-T₂, f_m is the measured fluorescence intensity of the sample added 3,3'-T₂, and f_r is the rest of the fluorescence intensity not quenchable with 3,3'-T₂. pH measurements were made with a TOA pH meter HM-20 and ultraviolet absorption measurements were in a Hitachi 557 double-beam spectrophotometer.

Results and Discussion

The pK of the phenolic hydroxyl group of 3,3'-T₂ was determined as 8.40 from the pH titration of the absorbance at 325 nm (data, not shown). This value is nearly the same as the pK of 8.45 for T₃.¹³⁾ In many reports, the importance of the contribution of the phenolic hydroxyl group of T₄ or T₃ to their binding to carrier proteins in serum, nuclear thyroid hormone receptor or iodothyronine mono deiodinase has been pointed out,^{2,14)} because the phenolic hydroxyl group could act as a hydrogen bond donor or an acceptor. Further, the anionic phenolate group could be involved in electrostatic interaction between thyroid hormones and their carrier proteins.^{6,15)} By this reason, the binding ability of 3,3'-T₂ to BSA was investigated at various pH's. Figure 1 shows the typical fluorescence quenching titration curve of BSA with 3,3'-T₂. The tryptophanyl fluorescence intensity of BSA was decreased by the addition of 3,3'-T₂ as a radiationless energy transfer,⁶⁾ while only

(1) Fig. 1. Relative Fluorescence Intensity of BSA vs. Molar Ratios of 3,3'-T₂ to BSA in 0.1 M Sodium Phosphate Buffer, pH 8.0 at 25 °C

(2) BSA concentration was 1.80 μM. Dotted line indicates the titration of the amino acid, tryptophan (1.80 μM) with 3,3'-T₂. Fluorescence excitation and emission wavelengths were 280 nm and 340 nm, respectively.

TABLE I. Parameters of Binding of 3,3'-T₂ to BSA at Various pH's at 25 °C

pH	Binding constant K (S.D.) $\times 10^{-5}$ (M ⁻¹)	Binding sites n (S.D.)
5.0	1.3 (0.1)	30 (5)
6.0	1.1 (0.1)	27 (6)
7.0	1.4 (0.1)	23 (6)
7.5	2.4 (0.2)	38 (6)
8.0	2.5 (0.1)	43 (9)
8.5	2.7 (0.2)	17 (5)
9.0	1.3 (0.2)	25 (8)

Standard deviations (S.D.) were obtained from ten times the fluorescence titrations at each pH.

the fluorescence of tryptophan was not affected. Although it is clear that there are multiple binding sites for given ligands having different binding affinities on the BSA molecule,¹⁶⁻¹⁸⁾ it is extremely difficult to determine each binding parameter from the fluorescence curves. Therefore, in this study, we calculated the apparent binding constants and the numbers of binding sites based on the assumption of the equivalence and independence of the binding sites. The calculated apparent binding constants and the number of the binding sites are summarized in Table I. These values are of the same order of magnitude within the pH range studied. A small increase of the apparent binding constant could be observed with increasing pH values and reached maximum at near pH 8.5. This pH is near the pK of the phenolic hydroxyl group of 3,3'-T₂ (pK=8.4). This result resembles the result that T₄, T₃ bound to human serum albumin (HSA) maximally at pHs corresponding to the pKs of their phenolic hydroxyl group of T₄ (pK=6.73) and T₃ (pK=8.5).¹⁵⁾ In the case of r-T₃ and 3-monoiodo-L-thyronine (T₁) binding to BSA,^{19,20)} binding also reached its maximum near the pKs of r-T₃ (pK=7.4) and 3-T₁ (pK=9.67), respectively. These results suggest that iodination of the 4'-hydroxyl group of the outer ring of the thyroid hormone metabolites plays an important role in strengthening the binding of these on the albumin molecule. The apparent binding constant of $2.4(0.06) \times 10^5 \text{ M}^{-1}$ at pH 7.4 is about one-eighth lower than the value of $1.16(0.4) \times 10^6 \text{ M}^{-1}$ at pH 7.4 for T₄. It resembles the value of $3.8(0.14) \times 10^5 \text{ M}^{-1}$ at pH 8.6 for T₃ or $4.43(0.15) \times 10^5 \text{ M}^{-1}$ at pH 7.4 for r-T₃, and is higher than the value of $8.85(1.07) \times 10^4 \text{ M}^{-1}$ at pH 7.4 for 3-T₁. A large number of binding sites on an albumin molecule for 3,3'-T₂ is considerably different from a few binding sites for T₄, T₃ or r-T₃^{15,19)} and resemble about 24 binding sites for 3-T₁.²⁰⁾ This result suggests the existence of a large number of non-specific binding pockets on the BSA molecule which fit the size for outer ring deiodinated or mono iodinated thyronine metabolites. The decrease of the binding constants above pH 8.5 could be explained by some alkaline conformational change of BSA.²¹⁾ The thermodynamic parameters for 3,3'-T₂ binding to BSA were also obtained at pH 8.0 by measuring the temperature dependence of the binding constant at a temperature range of 10–35 °C. The binding constant decreased with increasing temperature from $3.9(0.1) \times 10^5 \text{ M}^{-1}$ at 12.5 °C to $1.6(0.2) \times 10^5 \text{ M}^{-1}$ at 34.0 °C. The Van't Hoff plot is shown in Fig. 2. $\ln K$ is linearly related to $1/T$ in this temperature range. The

Fig. 2. Van't Hoff Plot of the Apparent Binding Constants at pH 8.0. Conditions were the same as in Fig. 1.

enthalpy change, ΔH^0 , was evaluated from the slope of the linear line as $-7.75 \text{ kcal mol}^{-1}$ at 25 °C. The free energy change, ΔG^0 , was $-7.36(0.02) \text{ kcal mol}^{-1}$ at 25 °C at pH 8.0 and the entropy change, ΔS^0 , was -1.51 e.u. at 25 °C. The free energy change of $-7.3(0.02) \text{ kcal mol}^{-1}$ resembles that of $-8.44(0.15) \text{ kcal mol}^{-1}$ for the binding of T₄ to BSA at pH 7.4 at 24 °C⁶⁾ or $-7.7(0.09) \text{ kcal mol}^{-1}$ for the binding of r-T₃ to BSA.¹⁹⁾ The principal contribution to the free energy binding of 3,3'-T₂ to BSA is derived from the large increase in the enthalpy term. The small decrease of the entropy is, however, very different from its large increase in T₄ binding to HSA (21–25 e.u.)¹⁶⁾ or r-T₃ binding to BSA (10.4 e.u.),¹⁹⁾ in which the entropy term is the main contribution to the free energy change for T₄ or r-T₃ bindings. The large negative enthalpy change and small negative entropy change could be partly explained by assuming rather hydrated water molecules on the 3,3'-T₂ bound BSA molecule, because the large increase of the entropy in T₄ binding to HSA could be accounted for by the release of water molecules from both HSA and T₄ molecules.¹⁶⁾

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