Thyroxine Binding Properties of Glycosylated Human Serum Albumin as Measured by Fluorescence

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Thyroid hormone, thyroxine (T₄), binding to glycosylated human serum albumin (G-HSA), and native human serum albumin (HSA) were studied as a function of pH using the fluorescence method. T₄ binding affinity for G-HSA was remarkably reduced in an alkaline pH as compared with the native HSA. The thermodynamic parameters for binding are estimated at pH 7.5: (a) for G-HSA, ΔG = −8.50 ± 0.04 kcal mol⁻¹ (30°C), ΔH = −5.2 kcal mol⁻¹, ΔS = +11 c.u.; (b) for HSA, ΔG = −8.89 ± 0.04 kcal mol⁻¹ (30°C), ΔH = −3.5 kcal mol⁻¹, ΔS = +18 c.u. These results suggest that the glycosylation of HSA causes a variation in the electrostatic interaction between T₄ and HSA.

Keywords thyroid hormone; thyroxine; hormone binding; human serum albumin; glycosylated human serum albumin; fluorescence

To date, the interaction of thyroxine (T₄) with non-defatted¹⁻¹⁰ or defatted¹¹⁻¹₃ human serum albumin (HSA) or bovine serum albumin (BSA) has been studied using various methods such as equilibrium dialysis, enzymatic, fluorescence or circular dichroism (CD) methods. The results of these studies indicate that there are one to two strong and several weaker binding sites on the albumin molecule. Tabachnick¹⁴ stated from the binding studies with acetylated and guanidinated HSA that positively charged ε-amino groups of lysine residues in HSA play an important role in the binding of T₄ to HSA at physiological pH; also, from the studies on the pH dependence of binding that the anionic phenolate group of T₄ potently attaches to the binding site on albumin. Based on Tabachnick’s proposition, in this study we aimed to clarify the effect of the glycosylation of HSA on the binding of T₄ for the following reasons: (a) Lys 525¹² and Lys 199¹³ of HSA have been identified as possible primary and secondary glycosylating sites for non-enzymatic glycosylation of HSA in vivo; (b) The results of this study will provide some fundamental information about T₄ circulation in the blood, especially its relation with the glycosylated albumin which is about 8% of the total albumin in the normal individual and an even greater percentage in the diabetic.¹⁴

MATERIALS AND METHODS

Human serum albumin (HSA) (lot No. 41) was obtained from Seikagaku Kogyo Co., Tokyo, and glycosylated HSA (lot No. 61H8090) was from Sigma Chemical Co., St. Louis. l-THyroxine (T₄) (lot No. M975936) was from Nakalai Tesque Co., Kyoto. Neither albumin was defatted, because most of the binding studies for the T₄-albumin system have been performed using native non-defatted albumin, and Tabachnick¹⁷ reported that the binding constants for the intact and the defatted HSA are completely the same at 6°C. Other reagents were of the highest quality available and obtained from Wako Pure Chemical Ind., Osaka.

The apparent binding constants were evaluated from the quenching data of the protein fluorescence with increasing T₄ concentration. The data were analyzed as in the previous studies¹⁷⁻¹⁸ according to Attallah and Lata,¹⁷ assuming the equivalence and independence of the binding sites,

\[ K = Q_s/(1-Q_s)(T_s-nQ_sP_s) \]

\[ Q_s = (f_r - f_m)/(f_r - f_l) \]

where \( K \) is the apparent binding constant, \( Q_s \) is the ratio of quenching at a point on the quenching curve to the maximum quenching, \( T_s \) is the total concentration of added T₄, \( P_s \) is the total albumin concentration, \( n \) is the number of T₄ bound to an albumin molecule, \( f_r \) is the total fluorescence intensity of an albumin solution without T₄, \( f_m \) is the measured fluorescence intensity of the sample in the presence of T₄, and \( f_l \) is the residual fluorescence intensity non-quenchable by T₄. The enthalpy change, \( \Delta H \) for T₄ binding to G-HSA and HSA were obtained from a van’t Hoff plot of the binding constant at a temperature range of 10—40°C according to the linear equation,

\[ \ln K = -\Delta H/\Delta R(1/T) + C \]

where C is a constant value.

The Gibbs free energy change, \( \Delta G \) and the entropy changes, \( \Delta S \) were also calculated using,

\[ K = \exp(-\Delta G/RT) \]

\[ \Delta S = (\Delta H - \Delta G)/T \]

Fluorescence measurements were performed with a Hitachi 850 spectrofluorometer. The temperature of the sample was controlled by the use of a hollow cell folder through which water from a constant temperature bath regulated within 0.1°C was circulated. Temperature in the cell was measured directly by a Takara thermister D641. The fluorescence excitation wavelength was 280 nm. In a typical experiment, aliquots (5—10 μl) of 0.2 mM T₄ were added to 1 ml of 5 μM albumin solution in 0.1 M sodium phosphate buffer at appropriate pHs. The observed fluorescence intensity was corrected to the concentration of the albumin. HSA and G-HSA concentrations were determined spectrophotometrically using \( E_{280nm}^{1cm,1%} = 5.3 \).
The molecular weights of HSA and G-HSA were assumed to be 66400 and 66800 respectively, according to the amino acid composition.14)

RESULTS AND DISCUSSION

The apparent binding constants for HSA and G-HSA were plotted as a function of pH in Fig. 1. The binding constant for HSA increased remarkably between pH 6 and 8. These results were similar to those reported by Tabachnick5 and Steiner et al.11) The binding constants for G-HSA are smaller than those for HSA at overall pH ranges, and the binding constant for G-HSA at pH 8.0 was only about one-third of that for the intact HSA. The remarkable increase in the binding constant for HSA at the alkaline pH has been explained by the ionization of the phenolic hydroxyl group of T₄ at these pH's (pK = 6.73) and the electrostatic interaction of the anionic phenolate group of T₄ with cationic lysine residues in HSA.5) Also, the primary and the secondary sites of non-enzymatic glycosylation of HSA have been identified as Lys 52512) and Lys 199,13) respectively. Shaklai et al.12) and Okabe and Hashizume18) reported that the glycosylation of HSA inhibits the binding of several drugs to HSA. These facts suggest that the glycosylation of HSA inhibits T₄ binding to HSA, especially in an alkaline condition, and the inhibition may be explained by the reduction of the electrostatic interaction between T₄ and lysine residues. The significance of the Lys 414 of HSA for the electrostatic interaction between T₄ and HSA has also been pointed out,14) and, in the crystal structure,19) Lys 525 is located not far from the active Lys 414. Therefore, Lys 525, as well as Lys 414, may also play an important role in T₄ binding to HSA. Figure 2 shows the van't Hoff plot of the apparent binding constants for T₄ binding to G-HSA and HSA at pH 7.5. The thermodynamic parameters are summarized in Table I. T₄ binding to G-HSA may be characterized by an increase in the Gibbs free energy change, and a decrease in the enthalpy and entropy changes. These results suggest that the glycosylation of HSA causes a variation in the electrostatic interaction between T₄ with HSA.

REFERENCES