Effect of Divalent Metal Ions on the Binding of Thyroxine to Bovine Serum Albumin as Measured by Fluorescence

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The binding of thyroxine (T₄) to bovine serum albumin (BSA) has been studied in the presence and absence of Ca²⁺, Cu²⁺ and Zn²⁺ ions at various pH's in 0.1 M Tris-acetate buffer at 25°C using the fluorescence method. In the presence of 50 μM Ca²⁺ and Zn²⁺ and the absence of metal ions, the binding constant (K) increased similarly with increasing pH values from pH 5 to pH 9, and the K value near midpoint, pH 7.4, was 1.66 ± 0.17 × 10⁸ M⁻¹. By contrast, the binding constant remained constant between pH 5 and pH 9 in the presence of 10 μM Ca²⁺, with an average value of 1.61 ± 0.22 × 10⁸ M⁻¹, suggesting a significant influence of Cu²⁺ ions on T₄ binding to BSA.

Keywords thyroid hormone; thyroxine; bovine serum albumin; binding constant; metal ion

Introduction

The interaction between thyroxine (T₄) or its metabolites and blood serum albumin has been studied extensively in terms of its biological importance.¹⁻⁶ In order to gain insight into the nature of the interaction of T₄ with serum albumin, pH dependence of the binding constants was studied by Tabachnick⁶ and Steiner et al.⁵ They found that the binding constants for T₄ to human serum albumin (HSA) showed a major increase in magnitude between pH 6 and pH 8. They explained their findings by attributing the increase of the binding constants to the electrostatic interaction between the negatively charged phenolic hydroxyl group of T₄ and the positively charged lysyl ε-amino group of HSA, since the phenolic hydroxyl group of T₄, pK 6.73, is approximately 82% ionized at pH 7.4.⁵

On the basis of their interpretation, it is considered that cationic metal ions bound on the serum albumin molecule may influence on the binding properties of thyroid hormone to serum albumin. Because HSA contains 9.42–9.98 mg/dl (0.236–0.249 mm) of Ca²⁺ ions, 73–149 μg/dl (12–23 μM) of Cu²⁺ ions and 84–159 μg/dl (13–24 μM) of Zn²⁺ ions,⁹ and about 50% of the Ca²⁺ ions, 10% of the Cu²⁺ ions and 70% of the Zn²⁺ ions are carried in the serum as a bound form to albumin.⁹ Therefore, in correlating this with in vivo biological importance, we aimed to investigate the effect of divalent metal ions, Ca²⁺, Cu²⁺ and Zn²⁺ ions, on the binding of T₄ to bovine serum albumin (BSA).

Materials and Methods

BSA (lot No. 86) was obtained from Seikagaku Kogyo Co., Ltd. T₄ (lot No. LKP0093), copper(II) acetate monohydrate, zinc(II) acetate dihydrate, calcium(II) acetate monohydrate, and other reagents of the highest quality were obtained from Wako Pure Chem. Ind., Osaka. Fluorescence measurements were performed with a Hitachi 850 spectrophotometer. 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 a Takara thermometer D641. The fluorescence excitation and emission wavelengths were 280 and 340 nm, respectively. In a typical experiment, aliquots (0.5–1.0 μl) of 0.2 mM T₄ were added to 200 μl of a 2 μM BSA solution in 0.1 M sodium phosphate buffer at various pH's in a 0.5 × 0.5 cm² quartz micro cuvette under stirring. The observed relative fluorescence intensity was corrected for dilution of albumin. The BSA concentrations were determined spectrophotometrically, using ε₁₀₀₀ = 6.54 at 280 nm⁴ and the molecular weight of 66 300⁸ in sodium phosphate buffer, pH 7.4. The apparent binding constants and the number of binding sites were evaluated from the fluorescence quenching titration curve of BSA with T₄ according to the method of Attallah and Lata,¹¹ assuming the equivalence and independence of the binding sites.

Results and Discussion

Figure 1 shows the fluorescence titration curves of BSA

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Fig. 1. Relative Fluorescence Intensity of BSA vs. Metal Ion Concentrations in 0.1 M Tris-Acetate Buffer, pH 7.4, at 25°C

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with Ca\textsuperscript{2+}, Cu\textsuperscript{2+} and Zn\textsuperscript{2+} ions. The relative fluorescence intensity of BSA was quenched about 30% by Ca\textsuperscript{2+}, Cu\textsuperscript{2+} and Zn\textsuperscript{2+} ions and reached a plateau when the metal ion concentrations exceeded about 25 \(\mu\text{M}\). These results indicate that the ionic atmosphere of the surroundings of tryptophan residues could be saturated by metal ions at metal ion concentrations above about 25 \(\mu\text{M}\). Figure 2 shows the typical fluorescence quenching curve of BSA obtained by titration with T\textsubscript{4} in the presence of 10 \(\mu\text{M}\) Cu\textsuperscript{2+} ions. The tryptophanyl fluorescence intensity decreased remarkably, reflecting T\textsubscript{4} binding to BSA. Similar quenching curves were also observed in the presence of both Ca\textsuperscript{2+} and Zn\textsuperscript{2+} ions (data not shown here). The effect of pH on the binding constants of T\textsubscript{4} to BSA obtained from the fluorescence titration curves in the presence of 50 \(\mu\text{M}\) Ca\textsuperscript{2+}, 50 \(\mu\text{M}\) Zn\textsuperscript{2+} and 10 \(\mu\text{M}\) Cu\textsuperscript{2+} ions, and in the absence of these metal ions, are shown in Fig. 3. The effect of pH on the binding constants for T\textsubscript{4} binding to BSA in the presence of both 50 \(\mu\text{M}\) Ca\textsuperscript{2+} and Zn\textsuperscript{2+} ions was similar to that in the absence of metal ions, and the binding constants increased with an increase in pH from pH 6 to pH 9. The binding constants at various pH's obtained in the absence of metal ions are summarized in Table I. The increases of the binding constants at an alkaline pH in the absence of metal ions were also similar to the case of T\textsubscript{4}-HSA, in which an increase

<table>
<thead>
<tr>
<th>pH</th>
<th>(K \times 10^{-6} ; (\text{M}^{-1}\text{ℓ}^{-0.9}))</th>
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<tr>
<td>5.0</td>
<td>0.83 ± 0.16</td>
</tr>
<tr>
<td>6.0</td>
<td>0.90 ± 0.05</td>
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<tr>
<td>7.0</td>
<td>1.22 ± 0.15</td>
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<tr>
<td>7.4</td>
<td>1.66 ± 0.17</td>
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<tr>
<td>8.5</td>
<td>1.90 ± 0.19</td>
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<tr>
<td>9.4</td>
<td>2.18 ± 0.06</td>
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\(a\) Standard deviations were obtained from five fluorescence titrations. \(b\) The best fit to the fluorescence data was obtained by setting the number of T\textsubscript{4} bound to a BSA molecule \((n) = 1\).

Fig. 2. Relative Fluorescence Intensity of BSA in the Presence of 10 \(\mu\text{M}\) Cu\textsuperscript{2+} as a Function of T\textsubscript{4} Concentrations. BSA concentration was 2.17 \(\mu\text{M}\). Other conditions were the same as in Fig. 1.

Fig. 3. Effect of pH on the Binding Constants for T\textsubscript{4} Binding to BSA in the Presence of Various Metal Ions. Conditions were the same as in Fig. 2. ○, metal free; ○, 50 \(\mu\text{M}\) Zn\textsuperscript{2+}; △, 50 \(\mu\text{M}\) Ca\textsuperscript{2+}; ▲, 10 \(\mu\text{M}\) Cu\textsuperscript{2+}.

Fig. 4. The Binding Constants \((K)\) and the Average Numbers of Binding Sites for T\textsubscript{4} Binding to BSA in the Presence of Various Concentrations of Metal Ions.

\(a\), Cu\textsuperscript{2+} (○, ■); \(b\), Zn\textsuperscript{2+} (□, □); Ca\textsuperscript{2+} (△, ▲). Open and closed symbols indicate \(K\) and \(n\), respectively. Conditions were the same as in Fig. 1.
of the binding constants at alkaline pH’s was explained by the electrostatic interaction between the ionized hydroxyl group of T₄ and the positively charged lysyl ε-amino group of HSA.²,⁶ Conversely, in the presence of 10 μM of Cu²⁺ ions, the binding constants remained constant between pH 5 and pH 9. The mean values of K in the presence of Cu²⁺ ions is 1.61 ± 0.22 × 10⁹ M⁻¹. In the presence of 50 μM Cu²⁺ ions, the binding constants could not be obtained from the present method because of the formation of a chelate complex consisting of T₄ and Cu²⁺ ions. These facts indicate that 50 μM of Ca²⁺ and Zn²⁺ ions did not affect the electrostatic interaction between the anionic hydroxyl group of T₄ and the cationic lysine residue, while 10 μM of Cu²⁺ did have an affect. The influence of Cu²⁺ ions on the electrostatic interaction between T₄ and the lysine residue of BSA. Both in the presence and absence of metal ions, the binding constants at pH 7.4 coincided well with each other and the binding constant is similar to 1.6 ± 0.4 × 10⁹ M⁻¹ for T₄-HSA.³ Figures 4a and b show the binding constants obtained at various concentrations of metal ions at pH 7.4, and 25 °C. All of them indicate that the binding constant remained constant even in the higher metal concentrations of this experiment. Average values of K were 1.60 ± 0.12 × 10⁹ M⁻¹ between 0—10 μM Cu²⁺, 1.60 ± 0.16 × 10⁹ M⁻¹ between 0—1.0 mM Zn²⁺ and 1.62 ± 0.1 × 10⁹ M⁻¹ between 0—1.0 mM Ca²⁺. On the other hand, the average number of T₄ binding sites in a BSA molecule increased from one to two in the presence of Cu²⁺ and Zn²⁺ ions as the concentrations of these metals increased, while the binding site was not affected by Ca²⁺ ions. Zgirski and Frieden¹² reported that at pH 7.4 one Cu²⁺ ion binds strongly to a BSA molecule with a K value of 1.1 × 10¹²—1.6 × 10¹³ M⁻¹, a second Cu²⁺ ion binds with a K value of 5.24 × 10⁶ M⁻¹, and three additional Cu²⁺ ions bind with a K value of 1.6 × 10⁶ M⁻¹. They also reported that the number of Cu²⁺ ions bound to a BSA molecule at pH 7.4 increased from one to two by increasing Cu²⁺ ion concentrations up to 5—10 μM. Their finding corresponds well with the increase of the number of T₄ binding sites in the BSA molecule observed in this study. Rao and Lal¹³,¹⁴) reported that a BSA molecule contains two primary binding sites to Zn²⁺ ions bound with log K = 3.9 at pH 6.5 at 30 °C. Österberg¹⁵) reported that one Zn²⁺ ion binds strongly to a BSA molecule with log K = 9.6 at pH 6.78 at 25 °C. These results suggest that one to two strong binding sites in a BSA molecule to Cu²⁺ and Zn²⁺ ions exist at a neutral pH. Adding to these reports, it has been also revealed that T₄ forms a firm chelate complex with Cu²⁺ ions.¹⁶,¹⁷ Although at the present time we have no knowledge about the Zn²⁺—T₄ chelate formation, Cu²⁺ and/or Zn²⁺ ions bound to a BSA molecule might play an important role in increasing the number of binding sites in a BSA molecule to T₄ at higher metal concentrations by forming chelate complexes with T₄. In the case of the BSA—Ca²⁺ system, the binding of Ca²⁺ ions to BSA could not affect the binding ability of T₄ to BSA, because the Ca²⁺ ion binding to the serum albumin is weak, with a K value of about 1.0 × 10² M⁻¹, and non-specific.¹⁸) Further, Ca²⁺ ions do not usually have a strong ability to form a chelate complex as compared with Cu²⁺ and/or Zn²⁺ ions.

References

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