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Thyroxine-Induced Conformational Changes in Prealbumin†

Gaetano Irace‡ and Harold Edelhoch*

ABSTRACT: The effects of thyroxine binding on the conformation of human prealbumin and bovine serum albumin have been examined. A blue shift in protein absorption was observed with prealbumin, whereas a red shift was observed with bovine serum albumin. In the case of prealbumin, where the two binding sites are identical, the total absorption change was confined to the binding of the first ligand and has been interpreted as resulting from a conformational change. A blue shift observed in the absorption spectrum of thyroxine, however, was the same for the first and second bound molecules. These data have been interpreted in terms of two identical and interacting sites on prealbumin and explain the origin of the difference in binding affinities between the first and second sites. Fluorescence quenching by thyroxine and thyroxine effects on tryptic hydrolysis of prealbumin are in accord with the above interpretation.

Prealbumin binds 2 mol of thyroxine (T_4)[1] or T_3 with binding constants that are two orders of magnitude different for each mole (Ferguson et al., 1975; Cheng et al., 1977). The negative cooperativity observed in the binding of the two hormone molecules is not due to heterogeneity in the binding sites, since these are identical as indicated from the X-ray analysis of Blake et al. (1971, 1974). Moreover, the closest approach of the two sites is almost 10 Å (Blake and Oatley, 1977), so that a steric mechanism cannot explain the interaction between the two sites. There are binding data at several pH values with the analogue DIPA, which suggests that an electrostatic interaction may contribute to the negative cooperativity (Cheng et al., 1977). A common mechanism of developing cooperativity in subunit proteins involves a conformational change on binding a ligand on one subunit, which then alters the interactions between subunits and, consequently, the binding affinity for subsequent ligands. We have examined the binding of T_4 and DIPA to PA in order to determine whether conformational changes are produced. For comparison, the binding of thyroxine to another thyroid hormone transport protein, i.e., bovine serum albumin, was also evaluated.

Materials and Methods

Human serum prealbumin (PA), obtained from Behring Diagnostics, was purified further by gel electrophoresis, as

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[1] Abbreviations used: PA, human serum prealbumin; BSA, bovine serum albumin; T_4, L-thyroxine; T_3, 3,5,3′-triiodo-L-thyronine; DIPA, 3-(4-hydroxy-3,5′-diiodophenyl)propionic acid; ANS, 8-anilino-1-naphthalenesulfonate; Tris, tris(hydroxymethyl)aminomethane.

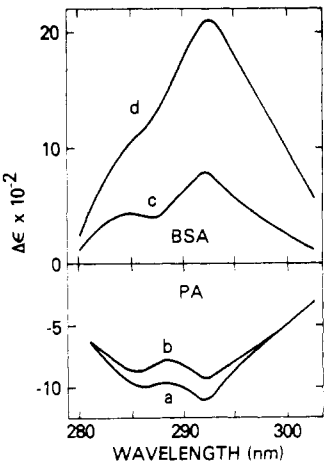

FIGURE 1: The effect of T_4 and DIPA on the ultraviolet difference absorption spectra of human PA and BSA: (a) PA-DIPA, $\bar{\nu}$ = 1.5; (b) PA-T_4, $\bar{\nu}$ = 1.5; (c) BSA-T_4, $\bar{\nu}$ = 0.87; (d) BSA-T_4, $\bar{\nu}$ = 1.65. Protein concentrations are listed in Figures 2, 3, and 5. Solutions contained 0.05 M Tris, 0.1 M NaCl at pH 8.6 and 25 °C.

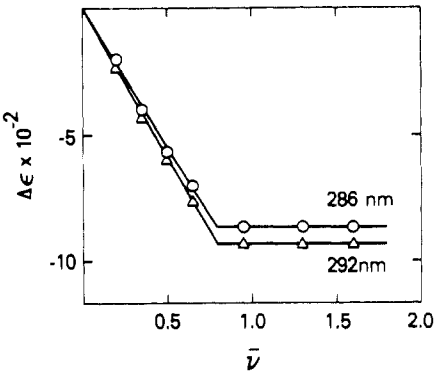

FIGURE 2: Difference titration of human PA. The ordinate is the difference absorption at 292 (△) and 286 (○) nm. The abscissa is the number of moles of T_4 bound per mole of PA. PA (14.9 μM) in 0.05 M Tris, 0.1 M NaCl, at pH 8.6, and 25 °C.

described by Cheng et al. (1975). PA concentration was determined by absorption at 280 nm ($E_{1cm}^{1\%}$ = 14.1) as described by Raz and Goodman (1969).

The sodium salt of T_4 was obtained from Calbiochem. Purity was checked by thin-layer chromatography on silica gel (Ogawara and Cahnmann, 1972) and by gas–liquid partition chromatography (GLC) (Funakoshi and Cahnmann, 1969). The concentration of T_4 was determined from its absorption in 0.01 M NaOH using a molar extinction coefficient at 325 nm of 6180 (Edelhoch, 1962).

DIPA and 4-hydroxy-3,5-diiodobenzaldehyde were prepared by the method of Matsuura and Cahnmann (1959), and their purity was confirmed by GLC, as above. The concentrations of DIPA and 4-hydroxy-3,5-diiodobenzaldehyde were determined in 0.01 M NaOH using the molar extinction coefficients reported by Pages et al. (1973): ϵ_{310} = 5500 for DIPA and ϵ_{342} = 20 200 for 4-hydroxy-3,5-diiodobenzaldehyde. Chemicals not mentioned above were reagent grade.

Ultraviolet Fluorescence. Fluorescence spectra and intensities were obtained with a Perkin-Elmer MPF-3A spectrofluorometer at 25 ± 1 °C. Fluorescence measurements were made in the range where emission was linear with protein concentration. The absorbance of all solutions was less than 0.1 OD at the excitation wavelength. Simultaneous readings were made of control solutions to correct for changes in lamp intensity or for fluctuations in photomultiplier response. Fluorescence titration curves were obtained by the addition of small increments of T_4 to 2 mL of PA solution. The observed relative intensities were corrected for PA dilution.

Ultraviolet Difference Spectroscopy. The tandem cell technique of Herskovits and Laskowski (1960) was used to measure difference absorption spectra with a Cary Model 14 spectrophotometer at 25 °C. Spectra were taken on PA solutions ranging between 14 and 16.5 μM.

Tryptic Digestion Rates. Digestion rates were measured with a Radiometer pH stat. The protein solution was kept at 25 °C under a stream of nitrogen and stirred continuously. Trypsin was dissolved in 0.001 N HCl containing 0.01 M $CaCl_2$ to a concentration of 4 mg/mL; 40 μL of trypsin was added to the reaction vessel containing 1.3 mL of 54.6 μM PA in presence and absence of 110 μM T_4. The small pH drop caused by the addition of trypsin was determined on an identical sample without PA. The pH of protein solutions was kept at pH 8.8 by continuous titration with 10^{-3} M KOH from an Agla syringe connected to the pH stat assembly. Tryptic digestions of thyroglobulin were made in absence and presence of different amounts of T_4 to show that T_4 has no effect on trypsin activity. Similar experiments were also performed with BSA (42 μM) using 2×10^{-3} M KOH as titrant solution.

Results

Absorption. Prealbumin. Knowledge of the three-dimensional structure of a protein can greatly facilitate the interpretation of perturbations in absorption spectra due to ligand binding or other interactions. The X-ray diffraction results with prealbumin reveal that neither the tryptophanyl nor the tyrosyl residues is part of the binding site for T_4 (Blake et al., 1974; Blake and Oatley, 1977).

The addition of T_4 to a solution of PA at pH 8.6 produced a blue shift in the absorption spectrum in the near-ultraviolet (Figure 1). The difference spectrum does not arise from ionized T_4, since its absorption spectrum shows a minimum at 294 nm. The small blue shift that occurs in the absorption of ionized T_4 with binding would not produce a difference spectrum characteristic of tyrosyl and tryptophanyl residues.

The negative peak of the difference spectrum at 292 nm arises from the indole groups in the protein (Donovan, 1969). The second, slightly smaller peak in the difference spectrum is at 286 nm. This peak probably can be assigned to tyrosyl residues, since the minor peak of the tryptophan perturbation spectra occurs at 284 nm (in 20% ethylene glycol) and is about half as intense as the major peak at 292 nm (Donovan, 1969).

The decrease in the difference absorption of the two peaks with an increase in the extent of site saturation is shown in Figure 2. We have used the two affinity constants (log K_1 = 8.5; log K_2 = 6.7) obtained by equilibrium dialysis of labeled T_4 at pH 7.4 (Ferguson et al., 1975) to calculate the number of T_4 bound/mol of PA. In order to see if the pH has much effect on the binding constants, we compared the binding of T_4 to PA by the ANS fluorescence method of Cheng et al. (1977). Very little difference was found between pH 7.4 and 8.6.

Another iodinated phenol, DIPA, which is strongly bound to PA and shows similar negative cooperativity, has also been evaluated (Figure 1). A similar negative difference spectrum was observed with DIPA as for T_4 at pH 8.6. A plot of the changes in absorption at the two peaks of the difference spectra against the extent of binding is shown in Figure 3. The two affinity constants, i.e., log K_1 = 8.5 and log K_2 = 5.3, determined by equilibrium dialysis at pH 8.6 (Cheng et al., 1977)

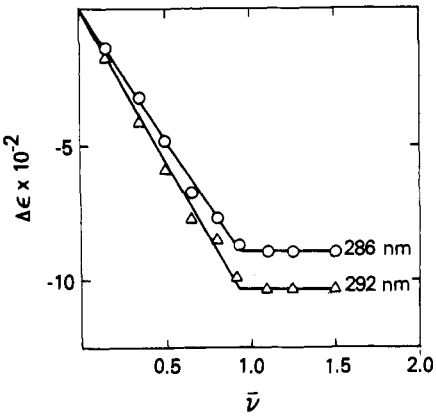

FIGURE 3: Difference titration of human PA. The ordinate is the difference absorption at 292 (△) and 286 nm (○). The abscissa is the number of moles of DIPA bound per mole of PA. PA (16.1 μM) in 0.05 M Tris, 0.1 M NaCl at pH 8.6 and 25 °C.

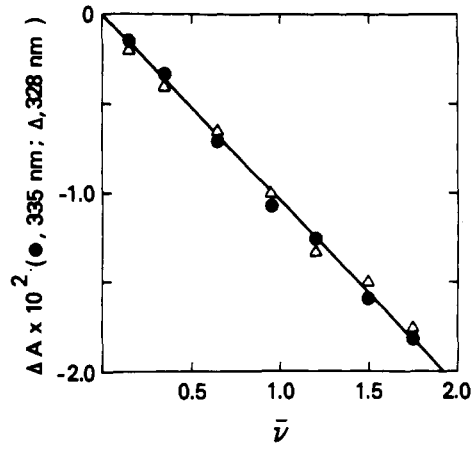

FIGURE 4: Difference absorption titration of T_4 and DIPA. The ordinate is the difference absorption of T_4 at 335 nm (●) and of DIPA at 328 nm (△). The abscissa is the number of moles of T_4 or DIPA bound per mole of PA. Solutions contained 14.9 μM PA with T_4 and 16.1 μM with DIPA. Solutions contained 0.05 M Tris, 0.1 M NaCl at pH 8.6 and 25 °C.

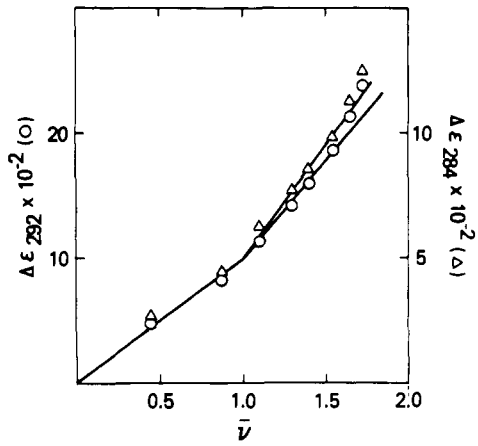

FIGURE 5: Difference titration of BSA. The ordinate is the difference absorption of BSA at 292 (○) and 284 nm (△). The abscissa is the number of moles of T_4 bound per mole of BSA. BSA (14.7 μM) in 0.05 M Tris, 0.1 M NaCl at pH 8.6 and 25 °C.

were used to calculate the number of moles of DIPA bound to PA.

With both T_4 and DIPA, a decrease in absorption is observed with only about half saturation of the two sites. Moreover, the magnitude of the effect produced ($\Delta A_{292}/A_{280}$) is very similar for both ligands, i.e., about 1%. A change of this size reflects some very minor rearrangement of the protein, since much larger absorption changes (i.e., $\Delta A_{285}/A_{280}$) are produced by acid (~5%) (Branch et al., 1971), thermally at pH 2.0 (~11%),[2] or by guanidinium hydrochloride denaturation (~15%) (Branch et al., 1972). The shape of the difference spectrum produced by the ligands is also different from that produced by pH, temperature, or guanidinium hydrochloride, since the ratio of the 292-nm peak to the 286-nm peak is greater than 1 with the two ligands and less than 1 with the denaturing conditions or reagents.

The binding of chromophoric ligands to proteins can produce a perturbation or shift in their absorption spectra. It has been reported that the binding of T_4 and DIPA produces a small (−2 nm) blue shift in their spectra without much change in their molar extinction coefficient (Pages et al., 1973). The decrease in the difference absorption peak of T_4 at 335 nm and of DIPA at 328 nm is shown in Figure 4. The absorption maxima for ionized T_4 and DIPA are at 325 and 310 nm, respectively. The negative absorption changes reported in Figure 4 for T_4 and DIPA, therefore, do not arise from a change in their ionization, since their ionization peaks occur at much shorter wavelengths than their difference peaks. In contrast to the effects of T_4 (or DIPA) on the perturbation spectrum of PA, the effects of PA on the perturbation spectrum of T_4 are the same for the first and second bound hormone molecules. This result is consistent with the earlier published analysis that PA contains two identical but interacting sites (Ferguson et al., 1975). A linear dependence of the difference absorption was also found for 4-hydroxy-3,5-diiodobenzaldehyde with binding to the two sites at pH 8.6. In this case, a much larger blue shift occurs (−6 nm), and the changes in the difference absorption are ten times as large as for T_4 and DIPA.

Bovine Serum Albumin. The binding affinities for the first two molecules of T_4 bound are about two orders of magnitude less than for PA (Steiner et al., 1966). There is no evidence, however, of a cooperative binding process with serum albumin. In contrast to PA, a red shift was observed with T_4 binding, and the peak at 292 nm was much larger than that at 284 nm (Figure 1). Moreover, the binding of the second molecule of T_4 produced almost twice the change observed with the first molecule (Figure 5). We have used values of the binding constants (log K) of 6.2 and 4.8 (Steiner et al., 1966) to calculate the number of moles bound at the first two sites. These values are for pH 7.4 but they do not change much by 8.6 (Steiner et al., 1966). The ratio of the two peaks does not change with binding to the two sites. In the case of BSA, both bands could originate exclusively from tryptophanyl residues, since the ratio of the two peaks is similar to that observed in the difference spectrum of tryptophan comparing water and 20% ethylene glycol (Donovan, 1969). Moreover, the BSA peak centered at 284 nm is in agreement with that found for the amino acid.

Fluorescence. The critical transfer distance (R_0) of the Förster theory (Förster, 1959) for transfer from tryptophan to ionized T_4 has been calculated to be 23.3 Å (Perlman et al., 1968). Consequently, quenching by energy transfer can be expected from the tryptophanyl residues situated near, but not in contact with, the binding site. The relative fluorescence quenching of PA with an increasing saturation of the two sites by T_4 is seen in Figure 6 when only the tryptophanyl residues are excited (i.e., excitation at 300 nm). The emission is quenched by 28% with binding to the first site, and no further quenching was observed with saturation of the second site.

[2] Unpublished experiments of the authors.

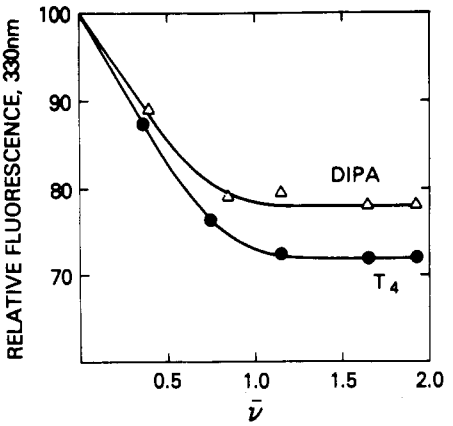

FIGURE 6: Degree of fluorescence quenching of human PA as function of T_4 (●) and DIPA (△) binding. The abscissa is the number of moles of ligand bound per mole of PA. PA (1.0 μM) in 0.05 M Tris, 0.1 M NaCl at pH 8.6 and 25 °C.

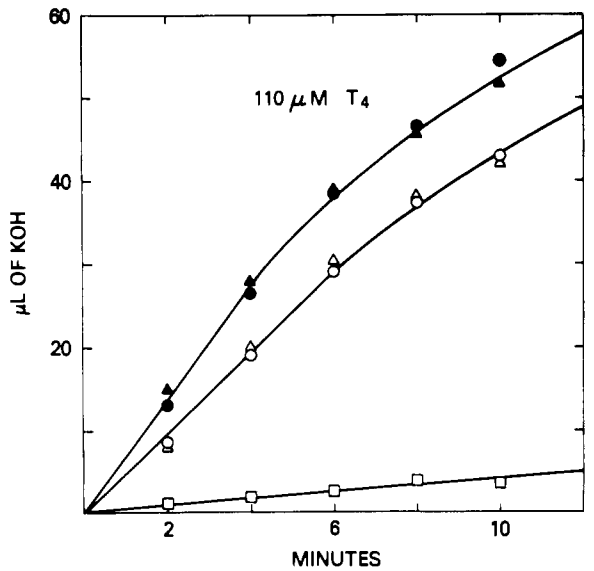

FIGURE 7: The effect of T_4 on the te of tryptic digestion of human PA (54.6 μM) at pH 8.8 and 25 °C: (n symbols, digestion in the absence of T_4; closed symbols, digestion ie presence of 110 μM T_4; (□-□) trypsin without PA or T_4. Titrant lution was 0.001 M KOH.

When excitation was at 280 nm, a small further decrease of about 5% was observed between one and two bound T_4 molecules, while the quenching produced by binding to the first site was essentially the same. One must assume that the fluorescence of all four of the typtophanyls located near the two binding sites (i.e., residue 79, coordinates 14.8, 9.7, and 3.9 for x, y, z axes from center of molecule) is completely quenched, since all of the quenching occurs with an occupation of the first site. This would appear to be rather unlikely, since two of these four tryptophanyl residues are much closer to the first binding site than to the second site. However, if the angles between the transition moments of tryptophanyl emission and T_4 absorption for the two tryptophans further away are much more favorable for energy transfer, larger values of k^2 would result, and the quenching of all four tryptophanyl residues can be explained. The alternative explanation is that the transfer that is observed with binding to the first site is lost with binding to the second site due to a conformational change produced by occupation of the first site; i.e., the angles between the transition moments become larger and preclude transfer.

The quenching of tryptophanyl fluorescence by DIPA has also been examined. The R_0 value for this compound should

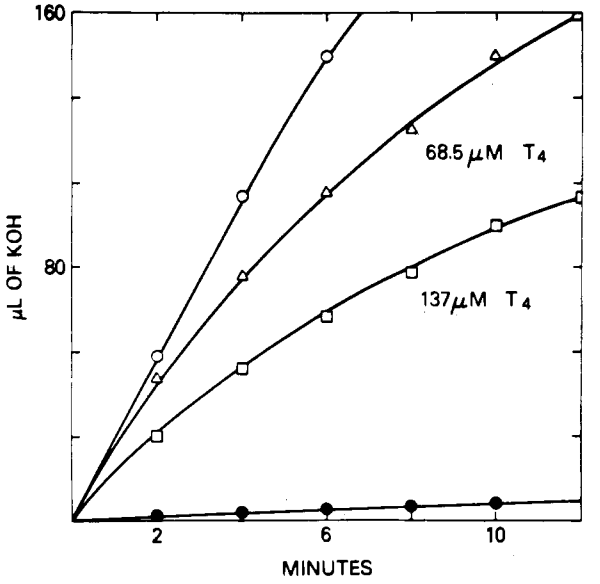

FIGURE 8: The effect of T_4 on the rate of tryptic digestion of BSA (42 μM) at pH 8.8 and 25 °C: (○-○) digestion in the absence of T_4; (△-△) digestion in the presence of 68.5 μM T_4; (□-□) digestion in the presence of 137 μM T_4; (●-●) trypsin without BSA or T_4. Titrant solution was 0.002 M KOH.

be less than that for T_4, since the overlap integral is less. The absorption peak of ionized T_4 is at 325 nm, while that for ionized DIPA is at 310 nm. In addition, the molar extinction coefficient of T_4 is about 20% larger, i.e., 6180 vs. 5500, at their respective peaks (Pages et al., 1973). In accord with the smaller critical distance the fluorescence of tryptophan was quenched only 20% by the first molecule of DIPA bound. The binding of the second molecule of DIPA also has no further effect on PA fluorescence.

Tryptic Digestion. The rate of tryptic hydrolysis of PA was measured with the binding sites empty and then almost fully occupied in order to see whether filling the sites changed the rate of hydrolysis. Since the initial rate of hydrolysis increases by almost one-third when 1.8 mol of T_4 was bound to PA, the binding must increase either the number of susceptible bonds or their rate of tryptic hydrolysis (Figure 7).

When the same reaction was performed with BSA, a significant decrease in the rate of tryptic hydrolysis was observed when 1.3 mol of T_4 was bound and a larger decrease occurred when 1.8 mol of T_4 was bound (Figure 8). T_4 binding clearly has a different effect on BSA than on PA hydrolysis. The decrease in rate for BSA and increase for PA can be explained if the red shift found for the former and blue shift for the latter protein with binding of T_4 result in a sequestration and exposure of residues, respectively, i.e., BSA becoming more and PA becoming less globular.

Discussion

PA is a tetrahedral tetramer of structurally identical subunits (Raz and Goodman, 1969; Gonzales and Offord, 1971; Morgan et al., 1971; Rask et al., 1971; Blake et al., 1971; Branch et al., 1971). Each chain is composed of two four-stranded β sheets. A dimer is formed by hydrogen bonds between β sheets to give two eight-stranded β-sheets. The association of two dimers forms a 16-stranded β cylinder about 50 Å long through the long axis of the molecule (Blake et al., 1974; Blake and Oatley, 1977). The channel formed by the two dimers has a diameter of about 8 Å, except it is constricted near the center. X-ray analysis of PA containing T_4 or T_3 indicates that the two hormone binding sites are located in the cylindrical

channel from ~4 to 16 Å from the center. Since the two sites are related by twofold symmetry, they are identical.

The difference absorption spectra of the aromatic chromophores produced by the binding of T_4 or DIPA on the first site of PA reveal that a conformational change takes place with binding. The blue shift in the spectrum cannot arise from the displacement of water molecules in the binding site, since none of the chromophoric side chains of tyrosine or tryptophan is located in the binding channel (Blake and Oatley, 1977). Moreover, a red shift is expected rather than a blue shift if the environment of tyrosine or tryptophan becomes less polar (Donovan, 1969). The blue shift indicates a rearrangement of the protein, resulting in an increase in the polarity of the environment of the two chromophores either by their greater exposure to the solvent or by new interactions with more polar residues.

It has been reported that a decrease in the polarity of the solvent by adding dioxane to water to 50% (v/v) produced a red shift of 6 nm in the absorption maximum of ionized T_4 and DIPA (Pages et al., 1973). The binding of T_4 and DIPA to PA, however, produces a blue shift of 2 nm (Pages et al., 1973). This result suggests that the observed shift is not due to a change in the polarity of these two ligands with binding. A blue shift would be expected, however, if the ionized hydroxyl group of the diiodophenolate moiety of the two ligands acts as an acceptor group in forming a hydrogen bond (Lees and Buraway, 1963). It has been proposed by Blake and Oatley (1977) that the phenolic hydroxyl group of T_4 is hydrogen bonded through a specific water molecule to Ser-117 and Thr-119.

The blue shift in T_4 and DIPA absorption is the same for the second molecule bound as for the first. The protein environment of the two sites, therefore, remains the same. Since the perturbation of the aromatic chromophores of PA is very different for the two binding sites, it appears that the binding of the first ligand produces the same conformational effect in both sites, thus maintaining the symmetry of PA when it contains ligand molecules. The conformational change is a relatively minor one, since the magnitude of the blue shift is very small compared to that observed by several other methods of unfolding the protein, i.e., acid, thermal, or guanidinium hydrochloride (Branch et al., 1971, 1972).

The quenching of tryptophanyl emission by the first ligand bound and not by the second supports the interpretation of the absorption data that a minor conformational change takes place. However, there is a second explanation which need not involve a structural change, but this is much less likely. The enhanced rate of PA hydrolysis by trypsin is also in accord with a minor conformational change which increases the exposure of susceptible bonds to the enzyme. The different types of data are, therefore, consistent with each other and can most readily be interpreted as reflecting a minor reorientation of residues or a slight unfolding of the native structure with ligand binding.

In the case of BSA, which has independent binding sites, the binding of T_4 produces a red shift in the absorption of the aromatic chromophores of the protein. Moreover, binding of T_4 to the second site has an even larger red shift than binding to the first site. Since the three-dimensional structure of BSA is not known, we cannot conclude that binding produces a conformational change because if the tryptophan residue(s) were part of the binding site a red shift would be expected. The large quenching of the tryptophan emission of BSA by binding of T_4 to the first site (Steiner et al., 1966) indicates that the residue(s) cannot be very far from the T_4 site. In fact, one could explain the quenching data if tryptophan was in the first site but if its transition moment for emission is at a slight angle to the transition moment of T_4 absorption.

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