

## FLUORESCENCE INVESTIGATIONS OF ALBUMIN FROM PATIENTS WITH FAMILIAL DYSALBUMINEMIC HYPERTHYROXINEMIA\*

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**Abstract**—Familial dysalbuminemic hyperthyroxinemia (FDH) is an autosomal dominant syndrome in which clinically euthyroid patients have elevated total thyroxine levels. These high serum thyroxine levels are traceable to altered binding of thyroxine to the patient's albumin. Albumin from FDH patients and normal volunteers have been purified. Reverse-phase and ion-exchange high performance liquid chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis on the FDH-human serum albumin (HSA) samples show a single band that comigrates with normal HSA. In both protein solutions the intrinsic fluorescence, upon 280 nm excitation, is predominantly due to the single tryptophan residue. The quantum yield of this intrinsic fluorescence in the FDH-HSA solutions is, however, reduced relative to that of HSA. Furthermore, the "average" lifetime value of the tryptophan emission in the FDH-HSA sample is less than that of normal HSA, consistent with its reduced quantum yield. The binding of thyroxine to both albumins effectively quenches the tryptophan emission probably *via* a nonradiative energy transfer mechanism. Time-resolved data suggest that the albumin from the dysalbuminemic patients is actually an approximately equimolar mixture of normal HSA and FDH-HSA indicative of heterologous expression. Quenching of the intrinsic HSA and FDH-HSA fluorescence by serial additions of thyroxine showed enhanced quenching of FDH-HSA relative to HSA at any T4 to albumin mole ratio, therefore supporting earlier reports of increased thyroxine affinity to FDH-HSA.

### INTRODUCTION

Familial dysalbuminemic hyperthyroxinemia (FDH)‡ is an autosomal dominant syndrome in which clinically euthyroid patients have elevated total thyroxine (T4) levels attributed to enhanced binding of thyroxine by the patients' albumin.<sup>1–3</sup> The FDH albumin has been the subject of a number of recent clinical investigations, which qualitatively confirmed the enhanced binding of T4.<sup>4–6</sup> The specific amino acid mutation and the molecular alterations underlying the increased thyroxine affinity, *e.g.* charged amino acid substitution and/or protein conformational change, remain to be determined.

Because human serum albumin (HSA) has a single tryptophan residue,<sup>7</sup> Trp 214, located in domain II, we considered that the intrinsic protein fluorescence could (1) be used to reveal possible conformational alterations between normal HSA and FDH-HSA and (2) be used analytically to monitor thyroxine binding to the albumin, thus facilitating the rapid acquisition of binding isotherms under equilibrium conditions. To this end we characterized some of the intrinsic fluorescence properties of normal HSA and FDH-HSA using both steady-state and time-resolved methodologies. The ef-

fects of thyroxine binding upon the tryptophan fluorescence were then studied at various protein concentrations.

### MATERIALS AND METHODS

*Source of sera.* Serum was obtained from a male patient diagnosed with FDH by methods previously described.<sup>8</sup> Serum was also obtained from a normal male volunteer.

*Purification of normal and FDH variant albumins.* The sera of the FDH patient and the normal volunteer were subjected to the same treatment. First, ammonium sulfate was added to the serum to 50% saturation, a condition wherein albumin is known to be soluble but many other plasma proteins are not. The pH of the supernatant from the ammonium sulfate fractionation was then lowered to the isoelectric point of albumin, pH 4.4, at 2–4°C. The precipitate was delipidated by reaction with Norit A-activated charcoal, at pH 3.0. The partially purified, delipidated albumin was dialyzed first against water and then against 0.05 M sodium phosphate buffer, pH 7.4. The albumin was further purified by DEAE-Sephadex chromatography using a linear gradient from 0.05 M sodium phosphate, pH 7.4, to 0.2 M sodium phosphate, pH 7.4. The major peaks were pooled, dialyzed against water and then lyophilized. Purity of the peaks was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).<sup>9</sup>

*Thyroxine titrations.* L-Thyroxine was obtained from Sigma Chemical Co. (St. Louis, MO) and used without additional purification. A stock solution of  $10^{-4}$  M L-thyroxine in 0.01 N NaOH was prepared; concentrations were determined using an extinction coefficient of  $6180 M^{-1} cm^{-1}$  at 325 nm.<sup>10</sup>

In a typical experiment, 360  $\mu$ L of a 3  $\mu$ M albumin solution in 0.14 M NaCl, 0.01 M sodium phosphate, pH 7.4, were placed in a short pathlength (3 mm  $\times$  3 mm) quartz cuvette and maintained at 37°C. The thyroxine stock solution was added in 2  $\mu$ L aliquots to the albumin solution to a final mole ratio of 3:1 thyroxine:albumin. For the initial albumin solution and after each thyroxine addition, the fluorescence intensity was measured at 340 nm, upon excitation at 295 nm.

*Steady-state spectroscopic measurements.* Absorption measurements were carried out using a Perkin-Elmer Lambda 5 Spectrophotometer. Fluorescence spectra were obtained using an SLM 8000C spectrofluorometer.

*Lifetime measurements and analysis.* Time-resolved data were

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‡Abbreviations: FDH, familial dysalbuminemic hyperthyroxinemia; FWHM, full width at half maximum; HSA, human serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; T4, thyroxine.

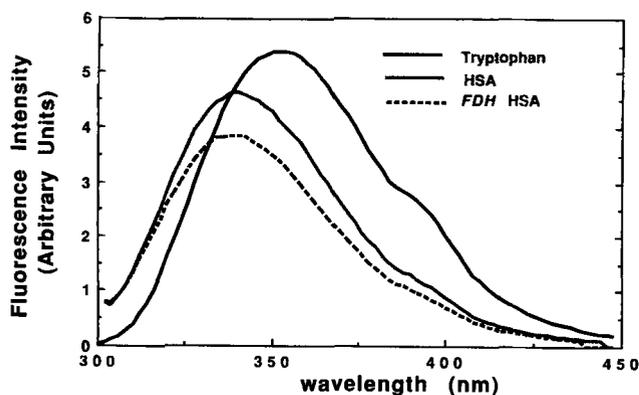


Figure 1. Emission spectra of normal and FDH-HSA and tryptophan.

obtained using an ISS Model K2 multifrequency phase and modulation fluorometer. The excitation source was the 300 nm region from a Spectra-Physics model 2045-3.0S laser; the exciting light was passed through a Corion 300 nm bandpass filter with a full width half maximum (FWHM) of 10 nm. Emission at wavelengths greater than 330 nm was viewed through Schott WG 325 cuton filters.

Phase and modulation lifetimes ( $\tau^P$  and  $\tau^M$ ) were calculated according to the equations<sup>11</sup>

$$\tan [P] = \omega\tau^P \quad (1)$$

$$M = [1 + (\omega\tau^M)^2]^{-1/2} \quad (2)$$

where P is the phase shift, M the relative modulation and  $\omega$  the angular modulation frequency. An emitting system characterized by a singly exponential decay will yield identical phase and modulation lifetimes irrespective of the modulation frequency. In the case of heterogeneous emitting systems (multiple noninteracting fluorescent species), the phase lifetimes will be less than the modulation lifetime. Furthermore, both of these apparent lifetimes will then depend upon the modulation frequency, namely, decreasing as the modulation frequency increases. The measured phase and modulation values are analyzed by assuming either a sum of exponentials<sup>12</sup> or by an alternative approach that involves the use of continuous lifetime distribution models.<sup>13</sup>

## RESULTS

### Spectroscopic measurements

Absorption spectra of normal HSA and FDH-HSA solutions were identical. Second derivative absorption spectra, which can be very sensitive to small spectral differences reflecting differential environments of tyrosine and tryptophan residues,<sup>14</sup> were also obtained for both samples but were also essentially identical.

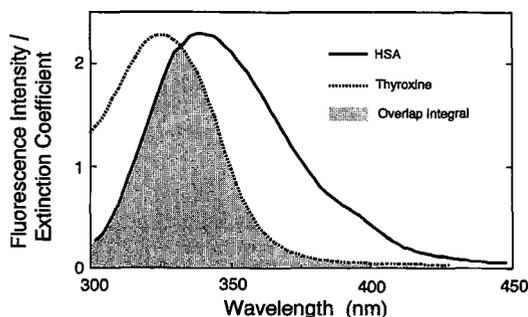


Figure 2. Emission spectra of HSA, excited at 295 nm, normalized to absorbance spectra of thyroxine with overlap integral.

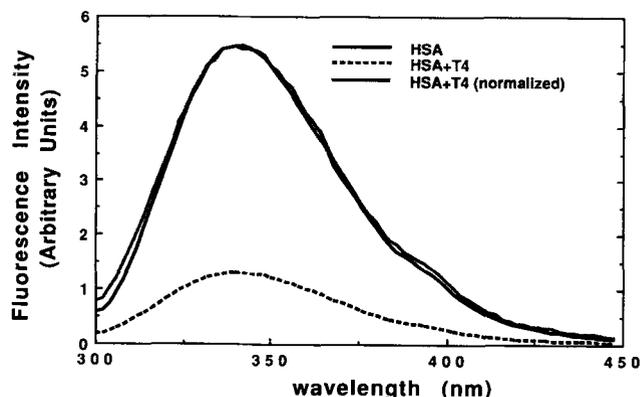


Figure 3. Emission spectra of 3  $\mu$ M HSA, excited at 295 nm, with and without a 2:1 mole ratio of thyroxine.

Uncorrected emission spectra of both albumin solutions (at 23°C), at the same concentration, are shown in Fig. 1. Although the emission maxima are identical (340 nm), the relative yield of the FDH-HSA was less than that of the normal HSA. Specifically, the quantum yields of the normal HSA and FDH-HSA solutions, excited at 295 nm, compared to tryptophan (assuming a value of 0.13 for the absolute quantum yield of tryptophan in water<sup>15</sup>) are 0.11 and 0.08, respectively.

The uncorrected emission spectrum of HSA, excited at 295 nm, normalized to the absorption spectrum of T4 is shown in Fig. 2. The absorption spectrum of T4 shows considerable overlap with the emission spectrum of HSA, suggesting that quenching can occur *via* a nonradiative energy transfer process. The actual transfer distance, in such a case, between donor and acceptor at 50% energy transfer efficiency,  $R_0$ , can be calculated according to  $R_0^6 = 8.78 \times 10^{-25} k^2 \Phi_0 J n^{-4}$  where  $n$  is the refractive index of the medium,  $k^2$  is the orientation factor between the transition moments of the donor and acceptor,  $\Phi_0$  is the quantum yield of the donor and  $J$  is the spectral overlap integral.<sup>16</sup> The overlap integral is calculated according to  $J = \int \epsilon(\nu)\epsilon(\nu)\nu^{-4}d\nu$  where  $\epsilon(\nu)$  is the HSA fluorescence spectrum normalized to unity and  $\epsilon(\nu)$  is the T4 absorption spectrum. Using a value of 1.5 for  $n$ <sup>17</sup> and a value of 2/3 for  $k^2$ ,  $R_0$  for T4-HSA was found to be approximately 20 Å.

The uncorrected emission spectrum of  $3 \times 10^{-5}$  M HSA, excited at 295 nm, with and without a 2:1 mole ratio of T4, is shown in Fig. 3. In the presence of T4, HSA exhibits a substantially reduced quantum yield, thus suggesting that T4 effectively quenches the excited tryptophan. These spectra were obtained in the short pathlength cuvette (3 mm  $\times$  3 mm) to minimize any potential inner filter effects. The emission spectrum of the HSA with T4 is also displayed normalized to the emission spectrum of HSA without T4 to illustrate the spectral invariance upon quenching. This spectral invariance supports an energy transfer quenching mechanism as opposed to trival reabsorption of the HSA emission.

### Thyroxine titrations

Quenching of the intrinsic HSA fluorescence by serial additions of thyroxine measured over a range of HSA concentrations is shown in Fig. 4. Relative fluorescence intensities of HSA as a function of the mole ratio of thyroxine to albumin

are displayed. Plots for 9  $\mu\text{M}$  and 30  $\mu\text{M}$  HSA were essentially superimposable suggesting that, at these protein concentrations, the stoichiometric binding regime was reached.

Quenching of the intrinsic fluorescence of 3  $\mu\text{M}$  HSA and FDH-HSA solutions by serial additions of T4 was measured and is shown in Fig. 5. The enhanced quenching of the FDH-HSA solution relative to HSA, at any T4 to albumin mole ratio, supports earlier reports of increased T4 affinity to FDH-HSA.

#### Lifetime measurements and analysis

Phase and modulation lifetime data were collected on both albumin samples over the frequency range of 15 MHz to 90 MHz at 25°C. The data were analyzed using discrete exponential or continuous distribution functions. The lifetime data on normal HSA fit best with a major discrete component (98%) of 5.75 ns plus a minor component (2%) attributed to scattered light (0.001 ns). By contrast, however, the lifetime data on the FDH-HSA solution were distinctly more heterogeneous. The data fit poorly to a single discrete exponential decay but improved markedly using either a continuous distribution model or a two-component analysis. Lifetime data on single tryptophan-containing proteins have generally shown that multiple exponential decays in such cases are the rule.<sup>18</sup> In this case, however, we were struck by the fact that the two component analysis yielded one component (5.72 ns) that was very close to the major component of normal HSA. We then carried out a global analysis on both data sets. Global analysis involves the simultaneous fitting of individual data sets having parameters in common.<sup>19</sup> In this case we linked one component in each data set and monitored the appearance of a second component. The linked component was 5.72 ns and accounted for 98% of the intensity in the HSA case and 70% of the intensity in the FDH-HSA case. In the FDH-HSA a second major component (29%) was observed as 3.01 ns. In both the HSA and the FDH-HSA case the reduced chi-squares were near two.

#### DISCUSSION

Single amino acid substitutions in a number of genetic variants of HSA (alloalbumins) have been characterized,<sup>20-22</sup> and these alloalbumins are rarely associated with disease or significant effect on physiological function.<sup>23</sup> In the case of the variant FDH albumin, the specific mutation is not known. Moreover, the FDH-HSA is electrophoretically identical to normal HSA, which complicates isolation of the mutant protein for subsequent amino acid sequencing. We undertook spectroscopic studies to determine if any conformational changes in the microenvironment of the tryptophan could be detected. As we shall discuss later, our time-resolved data give evidence for such a change.

The lifetime results on normal HSA, *i.e.* a major component near 5.75 ns, are reasonably consistent with previous lifetime studies. For example, van Hoek *et al.*<sup>24</sup> reported a heterogeneous decay for HSA that varied across the emission band; between 340 nm and 400 nm their average lifetime varied from 5.2 ns to 5.8 ns. The striking feature of our lifetime analyses is that the main (98%) component in the normal HSA case (5.75 ns) was also recovered in the case of FDA-HSA (5.72 ns), but in this second case it accounted for

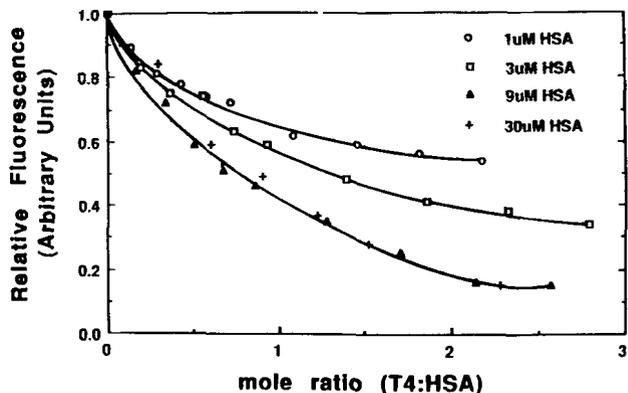


Figure 4. Quenching of the intrinsic HSA fluorescence (expressed as the ratio of the intensity in the presence of thyroxine to that in its absence) by serial additions of thyroxine measured over a range of HSA concentrations.

only 70% of the fractional intensity. The remaining fractional intensity (29%) was assigned to a 3.0 ns component. To convert the observed fractional intensities to molar concentration we may assume, to a first approximation, that the quantum yield of the tryptophan is proportional to its lifetime. Hence, if the lifetimes of these two species are in the ratio of 5.72/3.01, or 1.90, then the normalized fractional contribution of the short component, *i.e.* its molar concentration, is about 55%. Hence these results suggest that two albumin populations, with different lifetimes, are present in approximately equal proportions.

These considerations also suggest that the actual quantum yield of the FDH-HSA is not 0.08 but rather closer to 0.055, allowing for the fact that  $\sim 50\%$  of the protein is normal HSA with its higher quantum yield of 0.11. The calculated  $R_0$  value for tryptophan to T4 energy transfer (20 Å) is, of course, only approximate. We have not, for example, attempted to determine a more accurate value for  $k^2$ . Nonetheless, this value indicates that quenching could certainly occur *via* a Förster type mechanism. The observed average lifetime for the tryptophan decreased in both protein systems upon addition of T4 (data not shown), which also suggests a nonstatic quenching mechanism. The observed extent of quenching, 80% upon saturation, further suggests that the T4 binding site could be in the same domain (II) as the tryptophan residue.

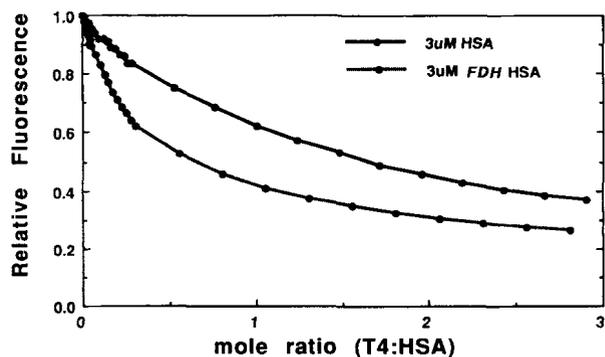


Figure 5. Quenching of the intrinsic fluorescence from 3  $\mu\text{M}$  FDH-HSA and normal HSA solutions (expressed as a ratio of the intensity in the presence of thyroxine to that in its absence) by serial additions of thyroxine.

The quenching curve of the FDH-HSA solution appears to contain two T4 binding components, a strong binding component and a weaker binding component. The weaker binding component is qualitatively similar to the single T4 binding component of the normal albumin solution. In the light of our lifetime data, we hypothesize that the FDH-HSA solution contains a roughly equimolar mixture of the FDH-HSA and normal HSA and that the respective components of the quenching curve of the FDH-HSA solution represent the major T4 binding site on each albumin. The weaker component binding thus represents the normal HSA T4 binding site, and the stronger binding component represents the tighter FDH-HSA T4 binding site.

Our hypothesis that the FDH serum contains a mixture of normal and variant albumins is compatible with the previous binding studies.<sup>5,6</sup> In both of these studies Scatchard analysis of equilibrium dialysis binding data from albumin purified from FDH serum revealed two-component T4 binding, one having the normal affinity constant and the other having an increased affinity constant. Barlow *et al.*<sup>5</sup> interpreted these results to indicate that the FDH serum contains an albumin that has an additional major T4 binding site. However, Lalloz *et al.*<sup>6</sup> suggested that it was reasonable to suppose from the two-component T4 binding and from the autosomal dominant pattern of FDH, that one of the two genes coding for albumin had been modified to produce a variant with increased affinity for T4 and that both genes were codominant. Yabu *et al.*<sup>25</sup> also suggested, on the basis of isoelectric focusing experiments, that FDH patients carried an overabundance of albumins with an increased affinity for T4. Our lifetime results support these observations and provide a means to quantify the codominance of normal and variant albumin in FDH serum. Our present efforts are directed toward using T4-bound affinity chromatography to investigate further this potential protein heterogeneity. The observed lifetime differences will serve as a convenient basis for distinguishing variant from normal HSA.

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