Mutations in a Specific Human Serum Albumin Thyroxine Binding Site Define the Structural Basis of Familial Dysalbuminemic Hyperthyroxinemia*

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The familial dysalbuminemic hyperthyroxinemia (FDH) phenotype results from a natural human serum albumin (HSA) mutant with histidine instead of arginine at amino acid position 218. This mutation results in an enhanced affinity for thyroxine. Site-directed mutagenesis and a yeast protein expression system were used to synthesize wild type HSA and FDH HSA as well as several other HSA mutants. Studies on the binding of thyroxine to these HSA species using equilibrium dialysis and quenching of tryptophan 214 fluorescence suggest that the FDH mutation affects a single thyroxine binding site located in the 2A subdomain of HSA. Site-directed mutagenesis of HSA and thyroxine analogs were used to obtain information about the mechanism of thyroxine binding to both wild type and FDH HSA. These studies suggest that the guanidino group of arginine at amino acid position 218 in wild type HSA is involved in an unfavorable binding interaction with the amino group of thyroxine, whereas histidine at amino acid position 218 in FDH HSA is involved in a favorable binding interaction with thyroxine. Neither arginine at amino acid position 222 nor tryptophan at amino acid position 214 appears to favorably influence the binding of thyroxine to wild type HSA.

Familial dysalbuminemic hyperthyroxinemia (FDH),[1] an autosomal dominant condition in which the total thyroxine level in serum is elevated while the free thyroxine level is normal, results from the presence of an abnormal human serum albumin (HSA) with an enhanced affinity for thyroxine (1). Although this condition had been widely reported in the medical literature (1–8), the molecular basis of FDH was not known until the identification of a single point mutation in the HSA gene of several FDH individuals resulting in the substitution of histidine for arginine at amino acid position 218 (9). This result was confirmed by another study in which the same mutation was identified in FDH individuals from eight unrelated families (10). Recently, it was shown that recombinantly produced FDH HSA has an enhanced affinity for thyroxine similar to that seen for natural FDH HSA (11), a result that confirmed that all of the information necessary to generate the FDH phenotype is contained in the FDH mutation.

The binding of thyroxine to HSA has been extensively studied (12–23), yet the molecular basis of this interaction remains obscure. Early studies used equilibrium dialysis to measure the binding of radiolabeled thyroxine and radiolabeled thyroxine analogs to HSA. Interpretation of these results was complicated by the observation of several binding components, which were difficult to resolve. For example, some of these studies assigned four equal binding sites for thyroxine with dissociation constants (K_d) of 6.6 μM (12–14), whereas other studies resolved the binding data into two sites with K_d values of 3.6 μM and six sites with K_d values of 25 μM (15–16). Data from other equilibrium dialysis studies were interpreted as fitting best to a multi-site model with one high affinity site (K_d value of 0.83 μM) and six lower affinity site (K_d values of 15 μM) (17).

The aforementioned results indicated that HSA has multiple thyroxine binding sites, whereas the existence of a specific thyroxine binding site in the 2A subdomain of HSA was suggested by other observations. Specifically, the 2A subdomain has been shown to be one of the two principal binding sites on HSA for small hydrophobic ligands (24–27). Binding studies with proteolytic HSA fragments have shown that the high affinity bilirubin binding site of HSA is located in the 2A subdomain (28). Other studies have shown that thyroxine competes with bilirubin binding at this high affinity bilirubin binding site, suggesting that the sites for these two ligands overlap (29).

HSA contains a single tryptophan residue at amino acid position 214, which is located in the 2A subdomain, and the fluorescence of this tryptophan is quenched by the binding of thyroxine (23, 30). This quenching has been exploited to measure the binding of thyroxine (23, 30), bilirubin (31), and a number of other 2A ligands (32–34). Studies measuring thyroxine binding to HSA by the fluorescence quenching method indicated a single high affinity site with a K_d value of 0.63 μM (23), in close agreement with the high affinity site (K_d of 0.83 μM) determined from equilibrium dialysis experiments (17).

To improve our understanding of the mechanism of thyroxine binding to the 2A subdomain of HSA, we used site-directed mutagenesis and a protein expression system to synthesize wild type HSA, FDH HSA, and several HSA mutants. The fluorescence quenching technique was used to measure the binding affinity of thyroxine and the thyroxine analogs, tetraiodothyroacetic acid (TA), 3,5,3′-triiodothyronine (T3), 3,5,3′-triiodothyropropionic acid (TP), and 3,3′,5′-triiodothyronine (RT3) to wild type HSA and to the HSA mutants (with the exception of a mutant in which leucine was substituted for tryptophan). The binding of thyroxine to wild type HSA and to the mutants was also measured by equilibrium dialysis. The following HSA mutants were synthesized: R218H (FDH) and R218M HSA substituting histidine or methionine for arginine at amino acid position 218, respectively; W214L HSA substi-

[1] The abbreviations used are: FDH, familial dysalbuminemic hyperthyroxinemia; HSA, human serum albumin; PBS, phosphate-buffered saline; TA, tetraiodothyroacetic acid; T3, 3,5,3′-triiodothyronine; TP, 3,5,3′-triiodothyropropionic acid; RT3, 3,3′,5′-triiodothyronine.

tuting leucine for tryptophan at amino acid position 214; R222M HSA substituting methionine for arginine at amino acid position 222. Recombinant wild type HSA was also produced.

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MATERIALS AND METHODS

Synthesis and Purification of Recombinant HSA

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Cloning of HSA Coding Region

With human liver cDNA as template, the entire coding region of the HSA gene including the native signal sequence was amplified by polymerase chain reaction using Vent DNA polymerase (New England Biolabs). The resulting DNA fragment was inserted into the plasmid vector pHiL-D2 (Invitrogen Corporation) using standard cloning techniques. pHiL-D2 is a shuttle vector that can be manipulated by cloning in *Escherichia coli* and that can also be used to introduce genes into the yeast species *Pichia pastoris* (Invitrogen Corporation) by homologous recombination. Specific mutations were introduced into the HSA coding region by using site-directed mutagenesis as described previously (11).

Expression of Recombinant HSA

Each pHiL-D2 expression plasmid contained a methanol-inducible promoter upstream of the HSA coding region. For each expression plasmid a yeast clone was isolated that contained the expression cassette stably integrated into the yeast chromosomal DNA. The native HSA signal sequence, which was left on the HSA coding region, directed high level secretion of mature HSA into the growth medium.

Purification of Recombinant HSA

The secreted HSA was isolated from growth medium as follows. The medium was brought to 50% saturation with ammonium sulfate at room temperature. The temperature was then lowered to 4 °C, and the pH was adjusted to 4.4, the isoelectric point of HSA. The precipitated protein was collected by centrifugation and resuspended in distilled water. Dialysis was carried out for 48 h at 4 °C against 100 volumes of distilled water, followed by 24 h against 100 volumes of phosphate-buffered saline (PBS; 150 mM NaCl, 40 mM phosphate, pH 7.4). The solution was loaded onto a column of cibacron blue immobilized on Sepharose 6B (Sigma) (35). After washing the column with 10 bed volumes of PBS, the HSA was eluted with 3 M NaCl. The eluent was dialyzed into PBS and passed over a column of Lipidex-1000 (Packard Instruments) to remove hydrophobic ligands possibly bound to the HSA (36). The resulting protein exhibited only one band on SDS-polyacrylamide gel electrophoresis.

Verification of DNA Sequence of HSA Clones

The total genomic DNA from each *P. pastoris* clone used to produce a particular HSA species was isolated using standard techniques. The genomic DNA isolated from each clone was used as template to amplify the entire HSA coding region by polymerase chain reaction. For each clone the entire HSA coding region was sequenced using the dideoxy chain termination technique, and the translation product corresponding to this sequence was found to match a previously published HSA sequence at all amino acid positions except for the mutation introduced into a particular HSA mutant (37).

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Fluorescence Quenching Studies

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Background

As previously shown (23, 30) the emission spectrum of HSA overlaps significantly with the absorption spectrum of thyroxine, suggesting that quenching of tryptophan 214 occurs via a nonradiative energy transfer process. The critical distance (R_o) for 50% transfer efficiency was estimated to be approximately 20 Å (30). Given the strong (sixth power) distance dependence of Förster type energy transfer (38), transfer can only occur when thyroxine is bound to HSA, *i.e.* free thyroxine does not contribute to the quenching. Moreover, given an R_o of 20 Å, we can assume that thyroxine molecules bound to the 2A subdomain, which contains tryptophan 214, are primarily responsible for the quenching. Hence, the net decrease in fluorescence of HSA upon serial additions of thyroxine is directly proportional to the fraction of HSA molecules with thyroxine bound.

To determine the dissociation constant for the thyroxine (or thyroxine analog)/HSA equilibrium, two separate experiments are required. Firstly, a high concentration of HSA is titrated with ligand to approximate, as closely as possible, stochiometric binding. In this case a plot of fluorescence *versus* the ligand/HSA ratio shows an initial monotonic decrease in fluorescence, which then plateaus at a minimum value reflecting the fraction of fluorescence not quenched by bound ligand. Secondly, a lower concentration of HSA is titrated with ligand, and the fraction of HSA molecules with bound ligand can be calculated knowing the quenching efficiency determined from the stochiometric binding isotherm described above. The K_d can then be calculated knowing the bound and free ligand concentrations at any HSA concentration. Protein concentrations were determined by absorbance at 280 nm using the 1-cm path length extinction coefficient $E^{1\%}$ of 5.3 (39) and by the Lowry method (the concentration of W214L HSA, which lacks tryptophan, was only determined by the Lowry method). The Lowry reagents purchased as a kit (Sigma) included a bovine serum albumin standard (concentration determined gravimetrically), which was used to generate a standard curve. Determinations by either method differed by less than 5%. Thyroxine and thyroxine analog concentrations were determined by the dry weight method.

Instrumentation and General Experimental Parameters

Fluorescence intensity measurements were made on an SLM 8000C spectrofluorimeter (SLM-Aminco, Champaign, IL) upgraded with ISS, Inc. (Champaign, IL) data acquisition hardware and software. Samples were excited at 280 nm with a 4 nm band pass, and emission at wavelengths longer than 300 nm was viewed through a Schott WG 305 cuton filter. All HSA samples were suspended in the buffer, 40 mM phosphate, pH 7.4, 100 mM NaCl, 0.3 mM EDTA (PBSE). The fluorescence intensity of the buffer blank was subtracted from all measurements. For all titrations, 800 μl of an HSA solution was placed in a dual path length fluorescence cuvette (10 × 2 mm) with the short path length oriented toward the emission side maintained at a temperature of 37 °C by a constant temperature circulator. After each addition of ligand, the sample was mixed using a pasteur pipette, and after 3 min the fluorescence intensity was recorded (the sample was not illuminated until the measurement commenced). All ligand stock solutions were prepared by dissolving the ligand at a concentration of 400 μM in 5 mM sodium hydroxide. Ligand stocks for some titrations were prepared by diluting concentrated stocks with distilled water.

Stochiometric Titrations

Wild type, R218H (FDH HSA), R218M, R222M, and W214L HSA were all treated identically. For the ligands thyroxine, TA, and RT3, a 10 μM HSA solution was titrated to a ligand/HSA mole ratio of four. Because of the lower binding affinity of T3 and TP, for HSA a 40 μM HSA solution was titrated with these ligands to a ligand/HSA mole ratio of four. Because W214L HSA does not contain tryptophan we used it as a control to determine to what extent the fluorescence due to the 18 tyrosine residues in HSA was quenchable by each ligand used in this study.

K_d Determinations

Wild type, R218H (FDH HSA), R218M, and R222M HSA were all treated identically. For thyroxine, TA, and RT3, 800 μl of a 0.4 μM HSA solution was titrated with the ligand. Because of the lower binding affinity of HSA for T3 and TP, a 3.0 μM HSA solution was titrated with ligand. The fraction of HSA molecules with a molecule of thyroxine bound at each point along the titration was taken as the fraction of the *quenchable signal* due to tryptophan 214 actually quenched at that point in the titration. For the binding of each HSA species to each of the ligands, the quenchable fluorescent signal due to tryptophan 214 was derived from the plateau region of the stochiometric quenching curve of that HSA species with that ligand as described below.

Experimental Corrections

Dilution and Inner Filter Effects—All fluorescence measurements were corrected for dilution and inner filter effects. Inner filter effects can arise due to either the absorption of the exciting light or by absorption of the emission by the ligands. These corrections were, in all cases, small (<5%) and were estimated for each ligand by the following method. 800 μl of a 10 μM L-tryptophan solution was added to the same cuvette that had been used for all of the fluorescent quenching experiments. The L-tryptophan solution was titrated with increasing amounts of ligand, and fluorescence intensity measurements were corrected for dilution. The assumption was made that there was no specific interaction between any of the ligands and L-tryptophan and that the reduction in the dilution corrected fluorescent intensity was entirely due to inner filter effects. These data could then be used to correct titrations of HSA with ligand for inner filter effects. 800 μl of a 180 μM L-tyrosine solution was also titrated with each ligand. The data from the titration of

L-tyrosine were used to correct titrations of W214L HSA with each ligand for inner filter effects.

Tyrosine Fluorescence—To avoid any contribution from tyrosine fluorescence, the HSA samples, ideally, should be excited at 300 nm or the fluorescence should be measured at wavelengths longer than 340 nm. The fluorescence signal obtained under these conditions, however, was too low to permit accurate intensity measurements with our instrumentation for some samples, and hence 280 nm excitation was utilized. The tyrosine contribution to the total fluorescence intensity of HSA and the HSA mutants is, however, small and can be estimated and accounted for in the following manner. Comparison of the fluorescence intensity of a 10 μM solution of W214L HSA, which does not contain tryptophan, to the fluorescence intensity of a 10 μM solution of wild type, R218H (FDH), R218M, and R222M HSA showed that the percentage of the total fluorescence due to tyrosine was 15, 20, 11, and 14% respectively. The fluorescence of a 10 μM solution of W214L HSA was found to be about 40% maximally quenchable for titrations up to a ligand/HSA mole ratio of four with the ligands thyroxine, TA, and RT3. The fluorescence of a 40 μM solution of W214L HSA was found to be about 65% maximally quenchable for titrations to a ligand/HSA mole ratio of four with the ligands T3 and TP. The value of the minimum relative fluorescence obtained at the plateau region in the stochiometric titrations of wild type, R218H, R218M, and R222M HSA with all of the ligands was corrected for the reduction in fluorescence due to the quenching of tyrosine fluorescence as follows. The percentage of the total fluorescence in each HSA solution due to tyrosine was multiplied by the fraction of that fluorescence determined to be quenched in a stochiometric titration of W214L HSA. This quenchable tyrosine fluorescence was added to the minimum relative fluorescence value experimentally obtained at the plateau region of a stochiometric titration of a particular HSA variant with a particular ligand to yield a corrected minimum relative fluorescence due only to the quenching of tryptophan. These data were then used to determine the fraction of the total fluorescent signal that was quenchable as a result of the quenching of tryptophan 214 fluorescence by the binding of ligand. Because the titrations used to determine the K_d values were carried out at much lower HSA and ligand concentrations, we assumed that the quenching of tyrosine fluorescence would be minimal for these titrations and made the assumption that all of the reduction in fluorescence intensity in these titrations was due to the quenching of tryptophan fluorescence.

Reversibility of Quenching

In order to demonstrate that the quenching of tryptophan fluorescence by thyroxine was reversible, the following experiment was carried out. Wild type, R218H, R218M, and R222M HSA were all treated identically. 800 μl of a 5 μM HSA solution was titrated with thyroxine to a ligand/HSA mole ratio of one. W214L HSA was then added to a W214L HSA/ligand mole ratio of one. The fluorescence due to tyrosine was assumed to be insignificantly quenchable in this range of thyroxine concentrations. Fluorescence intensity measurements were corrected for inner filter effects, for dilution, and for the increase in fluorescence due to the addition of W214L HSA. Relative total fluorescence values were converted to relative tryptophan fluorescence values by subtracting the tyrosine fluorescence from all intensity measurements. The expectation of this experiment was that W214L HSA would compete for thyroxine with the HSA species being titrated, reducing the quenching of tryptophan fluorescence.

Equilibrium Dialysis

A novel technique known as "waterbug" dialysis (40) was used to measure the binding of radiolabeled thyroxine to wild type, R218H, R218M, R222M, and W214L HSA. Equilibrium dialysis was carried out at 37 °C in PBSE. The cap from a 1.5-ml Eppendorf tube was used as a dialysis chamber. A small piece of dialysis membrane composed of regenerated cellulose with a molecular weight cut-off of 14,000 (Spectrum) was fastened over the open portion of the lid with a plastic ring obtained by cutting off the top of the Eppendorf tube from which the cap was obtained. 100 μl of a 10 μM HSA solution was added to a certain amount of radiolabeled thyroxine (DuPont NEN). The specific activity of radiolabeled thyroxine was 1280 μCi/μg. The resulting solution was then placed into the Eppendorf cap, and the chamber was sealed with a piece of dialysis membrane as described above. The sealed chamber, which was buoyant, was placed in a polypropylene tube containing 5 ml of a solution of unlabeled thyroxine of a certain concentration so that the dialysis membrane of the chamber was in contact with the 5-ml solution. The tube was incubated with gentle shaking for 24 h in an incubator maintained at 37 °C. After incubation a 50-μl sample was
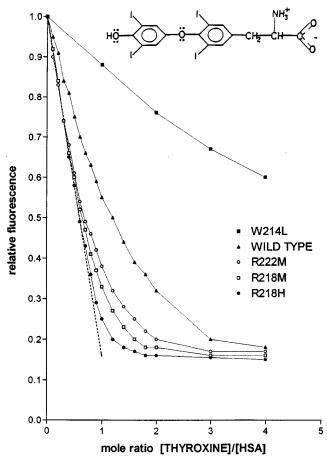

FIG. 1. **Stochiometric quenching titrations.** The *y* axis refers to the normalized fluorescence at each point along the titration. The *x* axis refers to the mole ratio of ligand to HSA (mol of ligand added/mol of HSA being titrated). A theoretical line corresponding to entirely stochiometric quenching (no ligand dissociation) for one binding site is shown as a *dashed line*. The structure of thyroxine is shown at the *top* of the graph.

removed from inside and outside the chamber, and both samples were counted in a γ counter. The free thyroxine concentrations ranged from 0.01 to 20 μM for all HSA species. For free thyroxine concentrations of 0.01 and 20 μM controls were run with no HSA added to the dialysis chamber to show that after 24 h a 50-μl sample from inside and a 50-μl sample from outside the chamber contained the same amount of radioactivity regardless of whether the radiolabeled thyroxine was initially added to the inside or outside of the chamber.

RESULTS

Cloning and Expression of HSA

All HSA species synthesized for this study were expressed at a level of approximately 500 mg/liter of induction medium. Recombinantly produced wild type and FDH HSA were found to be fully reactive with an antibody against authentic HSA. All purified recombinant HSA species were homogeneous as judged from denaturing polyacrylamide electrophoresis gels. All recombinant HSA species produced for this study ran at the same position as commercial HSA (Sigma) in denaturing electrophoresis gels. DNA sequencing confirmed that the DNA sequence of the HSA coding region derived from each of the yeast clones producing a particular HSA species was as expected.

Fluorescence Quenching Studies

Stochiometric Quenching—The normalized stochiometric quenching data for the binding of thyroxine to all HSA species is shown (Fig. 1). The *x* axis indicates the ligand/HSA mole
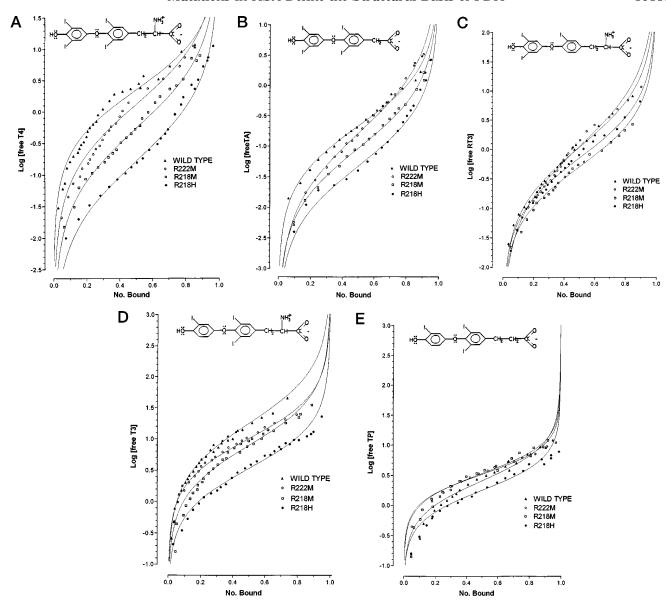
FIG. 2. **Binding isotherms corresponding to K_d values.** The binding curves derived from fluorescence quenching experiments for the ligands, thyroxine, TA, RT3, T3, and TP are shown in *A*, *B*, *C*, *D*, and *E*, respectively. The *y* axis shows the log of the free ligand concentration (μM). The *x* axis shows the number of ligand molecules bound/HSA molecule. The data points shown for the binding of a particular HSA species with a particular ligand represent an average data set derived from three separate titrations. The curve through the averaged data set corresponds to a theoretical binding isotherm with a K_d value and Hill coefficient, each determined by averaging the three values determined in the three separate titrations. The structure of the appropriate ligand is shown at the *top* of each graph.

ratio, whereas the *y* axis is the observed fluorescence. The stochiometric quenching curves of all HSA species for a particular ligand are quite similar. One notable exception is the stochiometric quenching curve for the binding of R222M to RT3, which is significantly different than that observed for RT3 binding to the other HSA species.

K_d Determinations—All titrations of a particular HSA species with a particular ligand were done three times. The fraction of HSA molecules with a ligand molecule bound (number bound) and the free ligand concentration were determined at each point along the titration. Each of the three data sets for each K_d determination were fit to the equation shown below by nonlinear regression (least squares method) using the computer program Prism (Graphpad).

Number bound $= 1/(1 + 10 \uparrow(\log K_d$

$$- \log \text{free ligand concentration}) * H)) \quad \text{(Eq. 1)}$$

The variable *H* is the Hill coefficient and is a measure of the

degree to which the relationship between the number bound and the log of the free ligand concentration deviates from simple binding. For simple binding with no positive or negative cooperativity the Hill coefficient is 1. In arriving at the best fit of the data to this equation, the computer varied both the K_d value and the Hill coefficient and reported a best fit value for both. Each K_d and Hill coefficient was determined by averaging the three K_d values and the three Hill coefficients determined in each of the triplicate titrations. These K_d values and Hill coefficients were used to generate a theoretical binding curve for the binding of each HSA species to each ligand. Because all three of the replicate titrations were done identically, an average data set was created by averaging the number bound and free ligand concentration at each point on the titration for all three data sets. This data set is shown along with the theoretical curve derived from all three data sets for each K_d determination (Fig. 2, *A–E*). K_d values and Hill coefficients for the binding of all HSA species to all ligands are compiled in Table

The average K_d value and Hill coefficient for the binding of each HSA species with each of the ligands are shown. The average values for both the K_d value and the Hill coefficient were determined by averaging the value obtained from three separate experiments. The values are shown ± S.D. The free energy value shown is the free energy of association of a particular ligand with a particular HSA species minus the free energy of association of wild type HSA with thyroxine. By definition the value for the binding of wild type HSA with thyroxine is 0.00.

	Thyroxine	TA	T3	TP	RT3
Wild type					
K_d (μM)	2.3 ± 0.6	0.22 ± 0.05	18.0 ± 2.8	2.6 ± 0.7	1.1 ± 0.3
Hill coefficient	0.93 ± 0.11	0.91 ± 0.10	1.00 ± 0.08	1.48 ± 0.18	0.79 ± 0.11
ΔG (kcal)	0.00	−1.44	1.26	0.08	−0.45
R222M					
K_d (μM)	1.4 ± 0.5	0.17 ± 0.06	10.0 ± 1.4	3.3 ± 0.1	1.1 ± 0.09
Hill coefficient	0.76 ± 0.13	0.71 ± 0.12	1.33 ± 0.09	1.89 ± 0.04	0.70 ± 0.03
ΔG (kcal)	−0.30	−1.60	0.90	0.22	−0.45
R218M					
K_d (μM)	0.54 ± 0.06	0.095 ± 0.029	7.9 ± 0.8	3.4 ± 0.4	0.51 ± 0.09
Hill coefficient	0.72 ± 0.10	0.82 ± 0.04	1.11 ± 0.18	1.77 ± 0.26	0.90 ± 0.15
ΔG (kcal)	−0.89	−1.96	0.76	0.24	−0.93
R218H					
K_d (μM)	0.17 ± 0.05	0.049 ± 0.014	3.4 ± 0.5	1.8 ± 0.3	0.71 ± 0.13
Hill coefficient	0.72 ± 0.08	0.83 ± 0.06	1.20 ± 0.04	1.66 ± 0.09	0.88 ± 0.10
ΔG (kcal)	−1.60	−2.36	0.24	−0.15	−0.72

I. Also, shown in Table I is a value equal to the free energy of association of a particular HSA species with a particular ligand minus the free energy of association of wild type HSA with thyroxine. This value represents the free energy difference resulting from a change in thyroxine and/or a change in the HSA binding site.

Reversibility of Quenching—The quenching of tryptophan fluorescence appears to be reversible for all of the HSA species examined (Fig. 3).

Equilibrium Dialysis

The data set obtained for the binding of thyroxine to each HSA species was fit to the equation shown below by nonlinear regression using the computer program Prism (Graphpad). This binding equation assumes two noninteracting binding components, each with a unique K_d value and a unique binding capacity.

$$\text{Number bound} = B_{max1} * X/(K_{d1} + X) + B_{max2} * X/(K_{d2} + X)$$

(Eq. 2)

B_{max1} and B_{max2} refer to the binding capacity of component one and component two, respectively, whereas K_{d1} and K_{d2} refer to the dissociation constants for component one and component two, respectively. The variable X represents the free thyroxine concentration. In an initial series of fits of the binding data for wild type HSA to the above equation, B_{max1} was held constant at 1, and K_{d1} was held constant at the value determined by the fluorescence quenching experiments described previously. In the first five fits of the data, the value of B_{max2} was held constant at the values 1, 2, 3, 4, and 5. The goodness of the fit improved as B_{max2} was increased from 1 to 5. Increasing B_{max2} from 5 to 10 improved the goodness of fit only slightly. Values of B_{max2} greater than 10 did not significantly improve the fit. Based on these results a value of 10 was arbitrarily chosen as a reasonable mathematical approximation for B_{max2}. Binding data for each HSA species was fit to the above equation with B_{max1} and B_{max2} held constant at 1 and 10, respectively. For each HSA species, K_{d1} was held constant at the value determined for the binding of that species to thyroxine in the fluorescence quenching experiments (Table I). For wild type, R218H, R218M, and R222M HSA, a best fit of the binding data to the above equation resulted in values for K_{d2} of 44 μM, 54, 50, and 48 μM, respectively. The average of these values for K_{d2}, 49 μM was used to determine an approximate value of K_{d1} for the binding of thyroxine to W214L HSA because Kd1 could not be
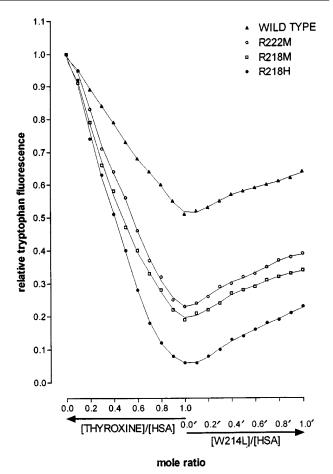

FIG. 3. **Reversibility of the quenching of tryptophan 214 fluorescence.** The *y* axis refers to the fraction of the initial tryptophan 214 fluorescence measured at each point along the titration. 0 to 1 along the *x* axis refers to number of mol of thyroxine added/number of mol of HSA being titrated. 0′–1′ refers to the mole ratio of W214L HSA added/mol of thyroxine. Initially, thyroxine was added to each HSA sample to a mole ratio of 1. This was followed by the addition of W214L to a mole ratio of 1.

determined using the fluorescence quenching technique, because W214L HSA does not contain tryptophan. The data for the binding of thyroxine to W214L HSA was fitted to the above equation with B_{max1}, B_{max2}, and K_{d2} held constant at 1, 10, and 49 μM, respectively. The value for K_{d1} determined in fitting the
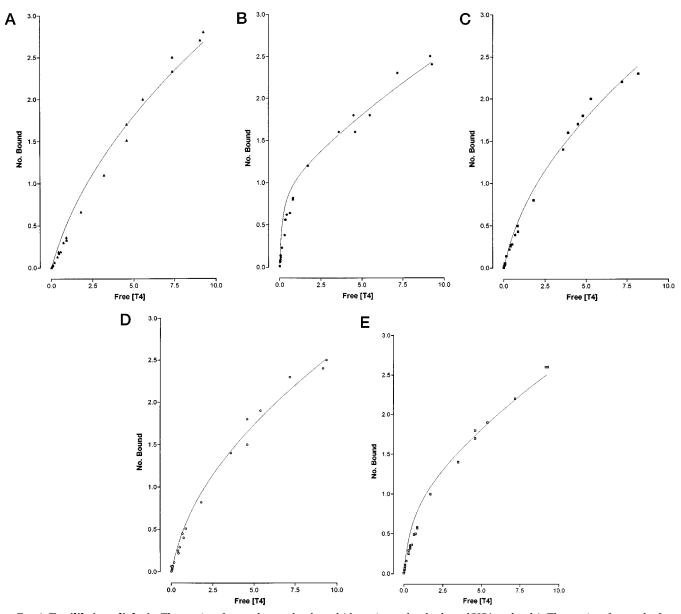
FIG. 4. **Equilibrium dialysis.** The y axis refers to the number bound (thyroxine molecules bound/HSA molecule). The x axis refers to the free thyroxine concentration (μM). *A, B, C, D,* and *E* refer to wild type, R218H, R218M, R222M, and W214L HSA, respectively. Each graph shows a data set obtained by combining the data from three equilibrium dialysis experiments. A theoretical curve corresponding to the best fit of the data to the two component equation discussed in the text is shown on each graph.

binding data for W214L HSA to the above equation was 1.5 μM. The equilibrium dialysis binding data and the theoretical curve corresponding to the best fit of that data to the two component equation are shown for each HSA species (Fig. 4, *A–E*).

DISCUSSION

The original hypothesis of this study, *i.e.* that the enhanced affinity of R218H (FDH) HSA for thyroxine results from changes in the structure of a specific high affinity binding site located in subdomain 2A, appears to be correct. A comparison of thyroxine binding data obtained by the fluorescence quenching and equilibrium dialysis techniques shows that introduction of specific structural changes (mutations) into subdomain 2A affected a single high affinity thyroxine binding site. A high capacity low affinity thyroxine binding component was not significantly affected by these mutations.

Using the equilibrium dialysis method alone, the high capacity low affinity thyroxine binding component is difficult to resolve from the specific 2A domain binding component. The presence of more than one binding component has confounded attempts to determine the molecular basis of thyroxine binding to HSA. The recently published x-ray crystallographic structure of HSA with many different hydrophobic ligands bound showed that in the crystal form binding occurs at two principal sites, the 2A and 3A subdomains of HSA (26, 27). Prior to the publication of this structure, it was shown that many HSA ligands displaced either of two fluorescent probes, which bound to distinct sites on HSA. The ligands could then be designated as site one or site two ligands depending on which probe they displaced (24, 25). The determination of the x-ray structure with a number of site one and site two ligands bound showed that site one ligands were bound to subdomain 2A and that site two ligands were bound to subdomain 3A. This evidence supports the idea that there are two principle binding sites on HSA for small hydrophobic ligands located in subdomains 2A and 3A.

The results of this study suggest that the enhanced affinity of FDH HSA for thyroxine partly results from removal of arginine 218. R218M HSA shows an increase in the free energy of

thyroxine binding of 0.9 kcal relative to wild type HSA, whereas R218H (FDH) HSA shows an increase of 1.6 kcal (Table I). We chose to substitute methionine for arginine because it most closely resembles arginine in size and shape while lacking the guanidino group. Specifically, we hypothesized that upon binding of thyroxine to wild type HSA an unfavorable interaction exists between the guanidino group of arginine 218 and the amino group of thyroxine. The binding of TA, a thyroxine analog that lacks the amino group, to wild type HSA is enhanced relative to thyroxine binding. The difference in the free energy of binding between R218H (FDH) and wild type HSA is significantly less for the thyroxine analog TA than for thyroxine itself. This finding suggests that the amino group of thyroxine has a more unfavorable interaction with the 2A binding pocket of wild type HSA than with the 2A binding pocket of R218H (FDH) HSA. This situation could result if there is an unfavorable interaction between the guanidino group of arginine 218 and the amino group of thyroxine in the binding of thyroxine to wild type HSA.

It has been shown by previous binding studies and in our study that the binding of T3, a thyroxine analog lacking an iodine atom on the outer phenyl ring to wild type HSA is reduced relative to thyroxine (12, 14–16, 23). This reduction has been shown to be partly due to an increase in the pK_a value of the ortho phenoxy group, which is believed to form a favorable electrostatic interaction with a positively charged amino acid such as arginine or lysine. Both R218M and R218H (FDH) HSA exhibit a higher affinity for T3 than wild type HSA with increases in the free energy of binding of 0.46 and 1.02 kcal, respectively. The binding affinity of wild type HSA for TP, a T3 analog lacking an amino group is greater than for T3. As described for thyroxine and TA above, the difference in the free energy of binding between R218H (FDH) and wild type HSA for TP is less than the difference for T3 binding. This result supports the idea that the interaction responsible for the enhanced affinity of R218H (FDH) and R218M HSA for thyroxine is separate and distinct from the interaction that causes a reduced affinity of wild type HSA for T3.

R218H (FDH) HSA has a higher affinity than R218M HSA for both thyroxine and TA, suggesting that specific characteristics of histidine may contribute to the enhanced affinity of R218H (FDH) HSA for thyroxine. Because the amino group of thyroxine is likely to be located near amino acid position 218, it was hypothesized that the inner phenyl ring of thyroxine adjacent to the amino group of thyroxine might directly interact with histidine at position 218. We measured the binding of wild type, R218H (FDH), and R218M HSA to the thyroxine analog RT3, which lacks an iodine atom on the inner phenyl ring. We found that the affinity of R218H (FDH) and R218M HSA for RT3 was nearly the same as the affinity of R218M for thyroxine, suggesting that both iodine atoms on the inner phenyl ring of thyroxine are required for maximum affinity binding of thyroxine to R218H (FDH) but not to R218M HSA. This finding supports the idea that there may be a specific interaction between the inner phenyl ring of thyroxine and histidine at amino acid position 218 in R218H (FDH) HSA for which both iodine atoms are required.

In this study we found that the substitution of leucine for tryptophan at amino acid position 214 did not reduce the affinity of HSA for thyroxine but instead increased it slightly. We found that the substitution of methionine for arginine at amino acid position 222 also increased binding slightly. In addition we found that the binding affinity of RT3 for wild type HSA increased relative to the binding of thyroxine. These observations suggest that the aromatic ring structure of tryptophan 214 and the guanidino group of arginine 222 do not favorably interact

with thyroxine. Also, both iodine atoms on the inner phenyl ring of thyroxine are not required for maximum affinity binding of thyroxine to wild type HSA. Interestingly, all of these three perturbations result in a slight increase in binding affinity. Although a solution of pure L-thyroxine exhibits no circular dichroism from 250 to 400 nm, it has been shown (20) that when L-thyroxine is bound to the high affinity site of wild type HSA there is an induced circular dichroism with peaks at 292 and 325 nm, due to the inner and outer phenyl rings of thyroxine, respectively. This induced circular dichroism indicates that thyroxine is held in a fairly rigid conformation when it is bound to the high affinity site of HSA. In this rigid conformation there are probably many steric constraints that interfere with optimal binding interactions. The two HSA mutants W214L and R222M HSA probably exhibit a slightly enhanced affinity for thyroxine because of a lessening of steric constraints imposed by the larger amino acids naturally present at amino acid positions 214 and 222. The increased affinity of RT3 for wild type HSA is probably also due to a lessening of steric binding constraints imposed by the large iodine atoms on the inner phenyl ring of thyroxine.

Because we assumed in our derivation of K_d values from fluorescence intensity measurements that the quenching of tryptophan 214 reports on the binding of one molecule of ligand to one site on HSA, the idea of cooperativity between sites is not consistent. In this case, then, the deviation of the Hill coefficient from unity can be thought of as a measure of the degree to which the curve that best fits the binding data deviates from an ideal shape. For all ligands except TP, the Hill coefficient ranges from 0.7 to 1.3. For TP binding to HSA the average Hill coefficient is 1.7, which represents more significant deviation from ideal behavior. The derivation of K_d values from fluorescence intensity measurements is an indirect method requiring many theoretical assumptions about the relationship between ligand binding and tryptophan 214 fluorescence. The inaccuracy of these assumptions may lead to systematic errors that are more significant for one ligand than for another. For example, we assume that the quenching of tryptophan 214 fluorescence results from a single binding event in subdomain 2A and that a high capacity low affinity binding component is not involved. This assumption is probably most accurate when there is a large difference between the K_d value for binding to the 2A subdomain and the K_d value of the high capacity low affinity component. In the case of TP binding to HSA, it seems possible that the K_d values for these two binding components are close enough so that binding due to both components occurs over a similar range of free ligand concentrations. If TP molecules are bound to sites other than the 2A subdomain yet close enough to tryptophan 214 to significantly quench its fluorescence, the additional quenching could result in a Hill coefficient significantly greater than one.

A recently published x-ray crystallographic structure of the ligand binding domain of a thyroid hormone receptor with triiodothyronine and several triiodothyronine analogues bound showed that there were no specific interactions between the amino group of triiodothyronine and the binding site of the receptor. This is similar to our finding that the amino group of thyroxine and triiodothyronine does not interact favorably with the thyroxine binding site of HSA (41).

Studies using recombinantly produced HSA mutants have allowed us to obtain more specific information about the binding of thyroxine to HSA than has been available previously. Presently, we are synthesizing other HSA species with mutations in the 2A subdomain of HSA in an attempt to further describe specific interactions between thyroxine and specific amino acid residues.

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