Mutagenesis Studies of Thyroxine Binding to Human Serum Albumin Define an Important Structural Characteristic of Subdomain 2A[†]

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ABSTRACT: 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. In our earlier study, site-directed mutagenesis and a yeast protein expression system were used to synthesize FDH HSA and several other HSA mutants. Measurement of the binding of these HSA mutants to thyroxine and several thyroxine analogs using equilibrium dialysis and quenching of tryptophan 214 fluorescence allowed us to propose a preliminary model of thyroxine binding to the 2A subdomain of wild type and FDH HSA. In this study, we have produced several other HSA mutants. By comparing the binding affinity of these mutants for thyroxine and tetraiodothyroacetic acid to the binding affinity of other mutants, we were able to suggest a new model for thyroxine binding to the 2A subdomain of HSA. We found that the substitution of arginine at position 218 with alanine increased the binding affinity for thyroxine by 2 orders of magnitude relative to the binding affinity of wild type HSA for thyroxine. A more accurate understanding of the mechanism of thyroxine binding to HSA has allowed us to define an important structural characteristic of subdomain 2A, one of the two principal binding sites on HSA for small hydrophobic ligands.

Familial dysalbuminemic hyperthyroxinemia (FDH[1]), an autosomal dominant condition in which the total thyroxine level 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 (Scottolini et al., 1984). This enhanced affinity of FDH HSA for thyroxine has been shown to be due to a single point mutation in the HSA gene causing a substitution of histidine for arginine at amino acid position 218 (Petersen et al., 1994).

In a previous study, recombinant wild type HSA, FDH HSA, and several other HSA mutants were synthesized using site-directed mutagenesis and a yeast protein expression system (Petersen et al., 1996). Binding of these HSA mutants to thyroxine and several thyroxine analogs was measured using equilibrium dialysis and the quenching of tryptophan 214 fluorescence. A comparison of the binding data obtained by equilibrium dialysis (which reports on all thyroxine binding to HSA) with fluorescence quenching data (which only reports on thyroxine binding to a specific site located in subdomain 2A) showed that the specific mutations introduced into subdomain 2A affected a single high-affinity thyroxine binding site, while a high-capacity low-affinity binding component was not affected.

It was suggested in our previous study that in the binding of thyroxine to wild type HSA there is an unfavorable interaction between arginine 218 and thyroxine and that the increased affinity of FDH HSA for thyroxine is not entirely due to specific characteristics of histidine at amino acid position 218 but is due mainly to the removal of arginine at amino acid position 218. The strongest support for this view was the finding that substitution of arginine 218 with methionine (R218M HSA) resulted in a mutant with a higher than normal affinity for thyroxine. It was also found that the thyroxine analog tetraiodothyroacetic acid (TA) which lacks an amino group has a higher binding affinity than thyroxine for wild type, R218M, and FDH (R218H) HSA. The observation that HSA mutants with increased binding affinity for thyroxine also exhibit a decrease in the difference between the free energy of binding of thyroxine and its deamino analog tetraiodothyroacetic acid suggests that the amino group on thyroxine contributes to an unfavorable interaction between thyroxine and arginine 218 in wild type HSA.

While these previous studies suggested that the amino group of thyroxine contributes to an unfavorable interaction between arginine 218 and thyroxine, the data were insufficient to determine if the interaction was primarily steric or electrostatic. We hypothesized that if the amino group of thyroxine is involved in an unfavorable electrostatic interaction with the guanidino group of arginine 218 that it might be possible to create a favorable interaction by substituting the negatively charged amino acid glutamate at position 218 (R218E). We also substituted two positions adjacent to position 218 [as shown in the crystal structure (Carter & He, 1992)], tryptophan 214 and arginine 222, with glutamic acid (W214E and R222E). The double mutants W214E/R218H and R218H/R222E were also synthesized. Alternatively, we hypothesized that the amino group of

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[1] Abbreviations: FDH, familial dysalbuminemic hyperthyroxinemia; HSA, human serum albumin; K_d, dissociation constant; PBS, phosphate-buffered saline (150 mM NaCl and 40 mM phosphate at pH 7.4); PBSE, phosphate-buffered saline with EDTA, 100 mM NaCl, 40 mM phosphate, and 0.3 mM EDTA; at pH 7.4); TA, tetraiodothyroacetic acid.

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thyroxine contributed to an unfavorable interaction with arginine 218 mainly by increasing the steric bulk of thyroxine. In order to test this hypothesis, we substituted arginine 218 with the small amino acid alanine (R218A).

Our results indicate that the FDH effect (dramatically increased binding affinity of FDH HSA for thyroxine) is due to removal of steric constraints imposed by arginine 218 on thyroxine binding to subdomain 2A. We found that a further reduction in these steric constraints obtained by substituting alanine for arginine 218 leads to an HSA mutant with an association constant for thyroxine approximately 1 order of magnitude greater than that observed for FDH HSA. Study of the structural basis of FDH HSA has fortuitously allowed us to define a potentially important role for arginine 218 in defining the structure of subdomain 2A, one of the two principal sites on HSA for binding of small hydrophobic ligands (Sudlow et al., 1975, 1976; Carter et al., 1992, 1994). Early views which held that HSA/ligand interactions are nonspecific and determined primarily by the lipophilicity of the ligand (Dunn, 1973; Fujita, 1972) are giving way to the mounting evidence that HSA/ligand interactions occur at two primary sites located in subdomains 2A and 3A and that these interactions are relatively specific. The finding that the association constant of HSA for thyroxine can be increased by 2 orders of magnitude by a single amino acid substitution in subdomain 2A would seem to support the latter view.

MATERIALS AND METHODS

Synthesis and Purification of Recombinant HSA

Introduction of Mutations into the HSA Coding Region. Specific mutations were introduced into the HSA coding region in a plasmid vector containing the entire HSA coding region (pHiL-D2 HSA) using standard techniques as previously described (Petersen et al., 1995).

Expression of Recombinant HSA. Each pHiL-D2 HSA expression cassette coding for a particular HSA mutant was introduced into the yeast species *Pichia pastoris* by electroporation. A yeast clone which contained the expression cassette stably integrated into the chromosomal DNA was isolated in each case.

Verification of the 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 a template to amplify the entire HSA coding region by the 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 (Minghetti et al., 1986) at all amino acid positions except for the mutation introduced into a particular HSA mutant.

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). The solution was loaded onto a column of cibracon blue immobilized on Sepharose 6B (Sigma) (Travis et al., 1976). After the column was washed 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 (Glatz & Veerkamp, 1983). The resulting protein exhibited only one band on SDS−PAGE.

Fluorescence Quenching Studies

Background. As previously shown (Dughi et al., 1993), 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 (Dughi et al., 1993). Given the strong (sixth power) distance dependence of Förster type energy transfer (Foster, 1948), 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 the thyroxine analog)/HSA equilibrium, two separate experiments are required. Firstly, a high concentration of HSA is titrated with ligand to approximate, as near as possible, stoichiometric 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. Second, 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 stoichiometric 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 (Sudlow et al., 1975) and by the Lowry method (the concentrations of HSA mutants lacking tryptophan were 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 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 mm × 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.

Stoichiometric Titrations. R218A, R218E, R222E, and R218H/R222E were all treated identically. A 10 μM solution of each HSA species was titrated with thyroxine and TA to a ligand/HSA mole ratio of 4. In these titrations, a plateau in the measured fluorescent intensity is reached at a ligand/HSA mole ratio of approximately 1. This fluorescence is assumed to represent the residual fluorescence when all HSA molecules have a ligand molecule bound, and from this value, the efficiency of quenching was calculated.

K_d Determinations. R218A, R218E, R222E, and R218/R222E HSA were all treated identically. Eight hundred microliter of a 0.4 μM HSA solution was titrated with the 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 stoichiometric quenching curve of that HSA species with that ligand as described previously (Petersen et al., 1996).

Experimental Corrections. Dilution of sample by addition of ligand, inner filter effects, and tyrosine fluorescence were all corrected for as described previously (Petersen et al., 1996).

Analysis of Data. 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 was fit to the equation shown below by nonlinear regression (least-squares method) using the computer program Prism (Graphpad).

$$\text{number bound} = 1/[1 + 10^{[(\log K_d - \log \text{free ligand concentration})H]}]$$

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 this case, the Hill coefficient is a measure of the degree to which the curve that best fits the data deviates from an ideal shape. In arriving at the best fit of the data to this equation, the computer program Prism (Graphpad) 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. Since 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 (Figure 1A,B). K_d values and Hill coefficients for the binding of all HSA species to all ligands are compiled in Table 1. Also shown in Table 1 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.

Equilibrium Dialysis

Experimental Procedures. A novel technique known as "waterbug" dialysis (Suter-Crazzolara & Unsicker, 1995) was used to measure the binding of radiolabeled thyroxine to W214E, W214E/R218H, R218H/R222E, R218E, R218A, and R222E 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 cutoff 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. One hundred microliters of a 10 μM HSA solution was added to a certain amount of radiolabeled thyroxine (New England Nuclear). 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 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 radiolabled thyroxine was initially added to the inside or outside of the chamber.

Analysis of Data. The data set obtained for the binding of thyroxine to each HSA species was fit to the equation shown below by nonlinear regression (least-squares method) 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.

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

B_{max1} and B_{max2} refer to the binding capacity of component 1 and component 2, respectively, while K_{d1} and K_{d2} refer to the dissociation constants for component 1 and component 2, respectively. The variable X represents the free thyroxine concentration. As previously described, a value of 10 was found to be a reasonable mathematical approximation of

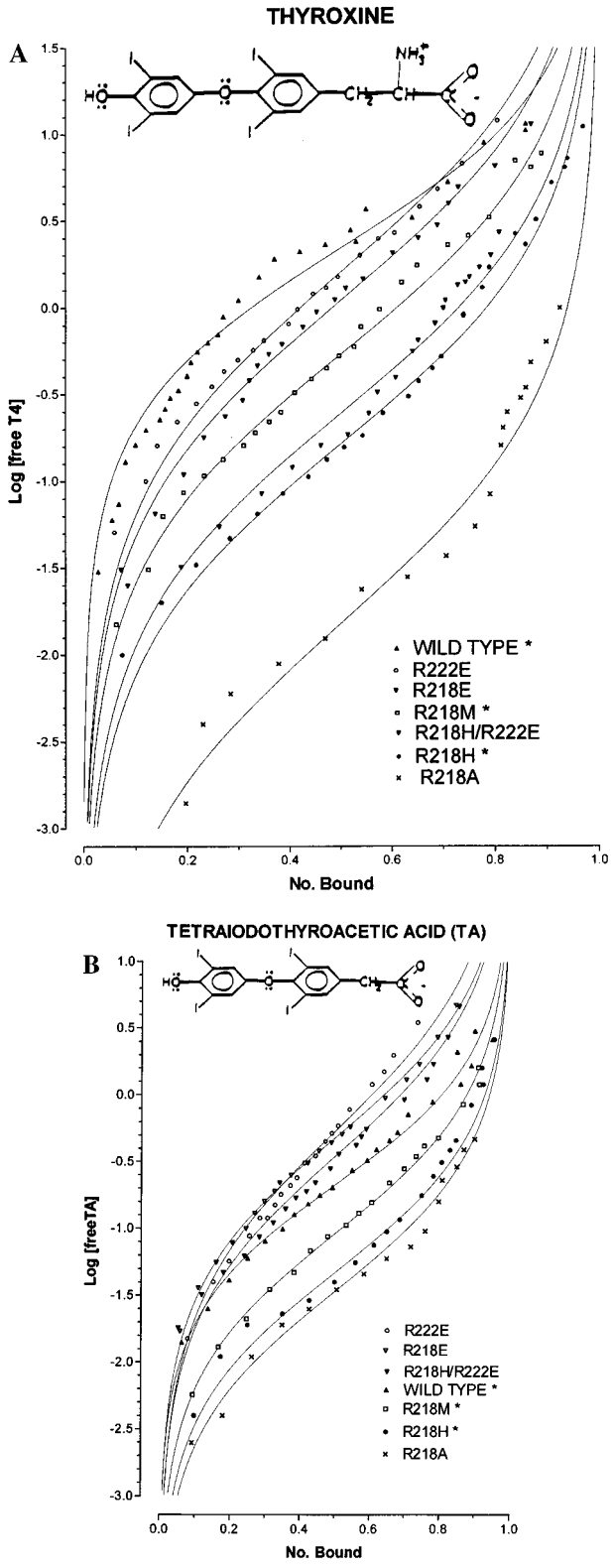

FIGURE 1: Binding isotherms corresponding to K_d values. The binding curves derived from fluorescence quenching experiments for the ligands thyroxine and TA are shown A and B, respectively. The *y* axis shows the log of the free ligand concentration (micromolar). The *x* axis shows the number of ligand molecules bound per 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. Asterisks denote data taken from Petersen et al. (1996).

Table 1: K_d Values and Corresponding Free Energy Differences Determined by Fluorescence Quenching[a]

		thyroxine	TA
wild type[c]	K_d (μM)	2.3 ± 0.6	0.22 ± 0.05
	Hill coeff	0.93 ± 0.11	0.91 ± 0.10
	ΔG (kcal)	0	−1.44
R222E	K_d (μM)	1.6 ± 0.3	0.54 ± 0.02
	Hill coeff	0.67 ± 0.08	0.68 ± 0.07
	ΔG (kcal)	−0.23	−0.89
R218E	K_d (μM)	1.1 ± 0.1	0.46 ± 0.03
	Hill coeff	0.70 ± 0.08	0.66 ± 0.03
	ΔG (kcal)	−0.46	−1.00
R218M[c]	K_d (μM)	0.54 ± 0.06	0.09 ± 0.029
	Hill coeff	0.72 ± 0.10	0.82 ± 0.04
	ΔG (kcal)	−0.89	−1.96
R218H[c]	K_d (μM)	0.17 ± 0.05	0.049 ± 0.014
	Hill coeff	0.72 ± 0.08	0.83 ± 0.06
	ΔG (kcal)	−1.6	−2.36
R218H/R222E	K_d (μM)	0.24 ± 0.02	0.35 ± 0.09
	Hill coeff	0.71 ± 0.09	0.74 ± 0.03
	ΔG (kcal)	−1.40	−1.17
R218A	K_d (μM)	0.015 ± 0.0020	0.034 ± 0.07
	Hill coeff	0.66 ± 0.06	0.82 ± 0.12
	ΔG (kcal)	−3.11	−2.61
W214E[b]	K_d (μM)	0.49[c]	
	Hill coeff		
	ΔG (kcal)	−0.96[c]	
W214E/R218H[b]	K_d (μM)	0.060[c]	
	Hill coeff		
	ΔG (kcal)	−2.26[c]	

[a] The average K_d value and Hill coefficient for the binding of each HSA species with each of the ligands are shown. The average value for both the K_d value and the Hill coefficient was determined by averaging the value obtained from three separate experiments. The value is shown ± one standard deviation. 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. [b] Approximate values determined from equilibrium dialysis experiments. [c] Values taken from Petersen et al. (1996).

B_{max2}, the binding capacity of the low-affinity high-capacity thyroxine binding component (Petersen et al., 1996). Binding data for R218A, R218E, R222E, and R218H/R222E HSA were 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 1). For R218A, R218E, R222E, and R218H/R222E HSA, a best fit of the binding data to the above equation resulted in values for K_{d2} of 50, 51, 73, and 62 μM, respectively. The average of these values for K_{d2}, (59 μM) was used to determine an approximate value of K_{d1} for the binding of thyroxine to W214E and W214E/R218H HSA since K_{d1} could not be determined using the fluorescence quenching technique, as these HSA species do not contain tryptophan. The data for the binding of thyroxine to W214E and W214E/R218H HSA were fitted to the above equation with B_{max1}, B_{max2}, and K_{d2} held constant at 1, 10, and 59 μM, respectively. 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 R222E, W214E, and W214E/R218H HSA (Figure 2A,C).

RESULTS AND DISCUSSION

Of our two alternative hypotheses which told that the unfavorable binding interaction between arginine 218 and

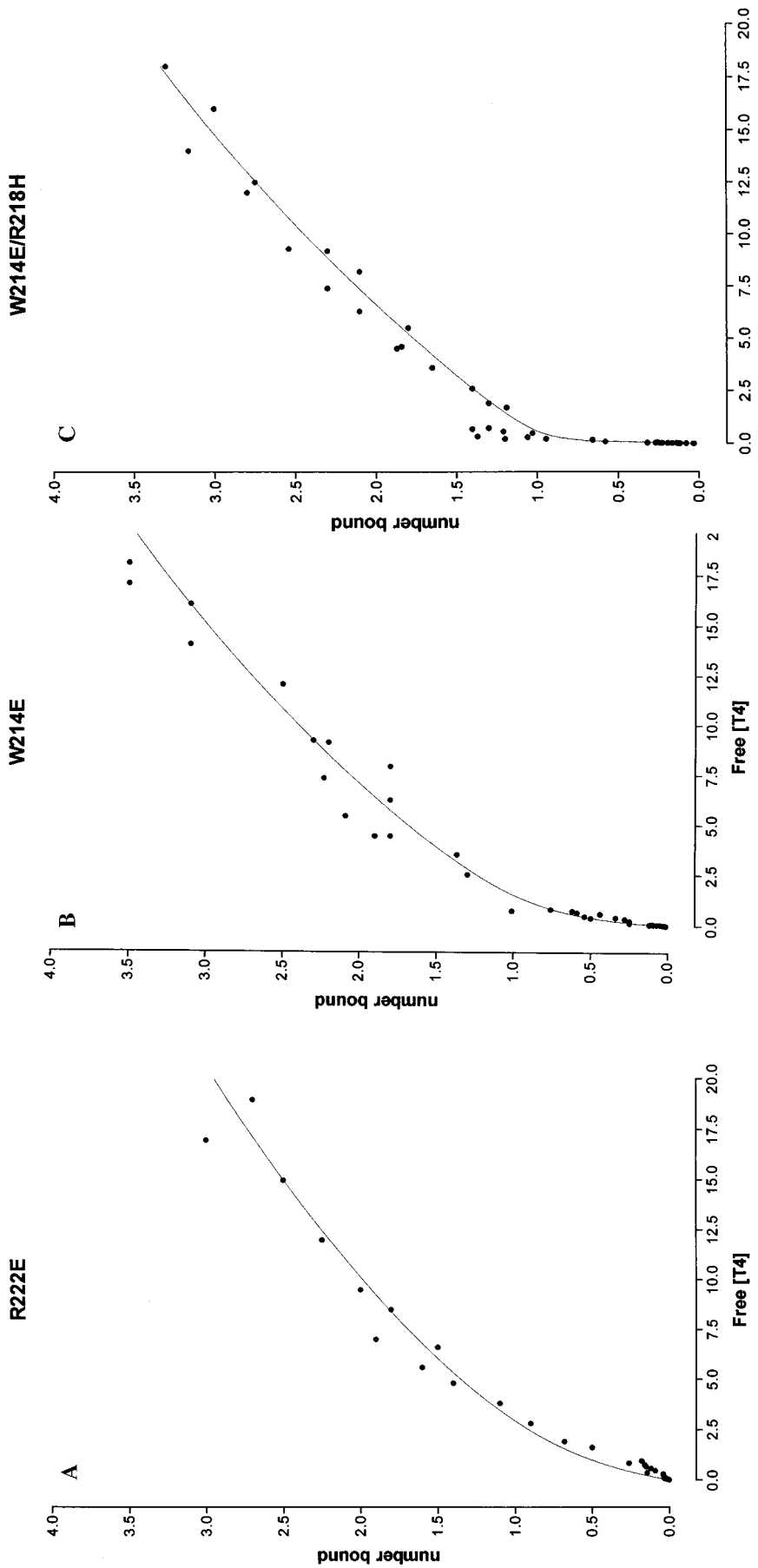

FIGURE 2: Equilibrium dialysis. The *y* axis refers to the number bound (thyroxine molecules bound per HSA molecule). The *x* axis refers to the free thyroxine concentration (micromolar). Panels A−C, refer to R222E, W214E, and W214E/R218H HSA, respectively. Each graph shows a data set obtained by combining 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 in each graph.

thyroxine is due to either electrostatic or steric forces, the latter appears to be correct. Substitution of arginine 218 with glutamate leads to only a slight increase in thyroxine binding affinity, contrary to our hypothesis that a favorable electrostatic interaction might be created by replacing the positively charged amino acid arginine with the negatively charged amino acid glutamate. Substitution of arginine 222 with glutamate had no significant effect on thyroxine binding. Substitution of tryptophan 214 with glutamate increased thyroxine binding affinity significantly. However, in a previous study, we showed that substitution of tryptophan 214 with leucine increased affinity for thyroxine (Petersen et al., 1996), suggesting that glutamate at position 214 could lead to increased binding by reducing unfavorable steric interactions. Further mutagenesis of amino acid position 214 is necessary to determine whether the substitution of tryptophan 214 with glutamate increases thyroxine binding primarily through a steric or electrostatic mechanism.

Substitution of arginine 218 with the small amino acid alanine increased binding affinity for thyroxine dramatically so that the association constant is more than 1 order of magnitude greater than that observed for FDH HSA (R218H). A comparison of the free energies of binding for thyroxine and its deamino analog TA compiled in Table 1 shows an important trend. As the free energy of binding to thyroxine increases in the series wild type, R218M, R218H, and R218A HSA, the difference in the free energy of binding between thyroxine and its deamino analog TA decreases. For R218A HSA, the difference in binding affinity between thyroxine and TA nearly disappears. For all the other HSA species in the series, TA has a binding affinity significantly higher than that of thyroxine. This trend in the data can be interpreted as follows. Arginine 218 exerts an unfavorable effect on the binding of thyroxine to the 2A subdomain of HSA through a steric effect. As the amino acid at this position is substituted with amino acids that exert a progressively smaller unfavorable steric effect, the binding affinity for thyroxine increases. The amino group of thyroxine enhances this unfavorable interaction by contributing to the steric bulk of thyroxine so that as the unfavorable steric interaction is reduced by mutation of the amino acid position 218 the difference between the affinity for thyroxine and TA is reduced. Substitution of arginine with alanine completely eliminates the unfavorable steric interaction between thyroxine and the amino acid at position 218 in the 2A binding site, leading to a maximum binding affinity for thyroxine and a nearly equal affinity for thyroxine and TA.

Measurements of the local motion of tryptophan 214 in the wild type, R218M, and R218H (FDH) HSA using time-resolved fluorescence methodologies have shown that the local motion of tryptophan 214 is greater in R218M and R218H (FDH) HSA than that observed for wild type HSA (Helms et al., 1996). The fact that these HSA mutants which exhibit enhanced affinity for thyroxine also exhibit greater local motion of tryptophan 214 suggests that the mechanism by which arginine 218 limits the motion of the adjacent amino acid tryptophan 214 and interferes with optimal thyroxine binding to the 2A subdomain of HSA may be similar. It seems likely that both mechanisms involve steric constraints imposed by arginine 218.

The presence of arginine 218 in the 2A subdomain of wild type HSA lowers dramatically the total serum thyroxine concentration of an individual from what would be expected if a smaller amino acid was naturally present at amino acid position 218 in wild type HSA. It is noteworthy that arginine 218 is well conserved in evolution [conserved in species as unrelated as frogs and humans (Carter & Ho, 1994)] which indicates a potential role for this amino acid in physiologically important HSA/ligand interactions. Arginine 410 is located in a position in subdomain 3A homologous to arginine 218 in subdomain 2A (Carter & Ho, 1994). As observed for arginine 218, arginine 410 is also highly conserved in evolution. One might speculate that arginine 218 and arginine 410, in subdomains 2A and 3A, respectively, act as gatekeepers by reducing the affinity of certain ligands and that this is related to their conservation in evolution. Determining the mechanisms by which the two principal sites on HSA interact with a wide variety of structurally unique ligands is an active area of research. Our results suggest that defining the role of arginine 218 in HSA/ligand interactions may be important in understanding the structure of subdomain 2A. We are currently measuring the binding of several subdomain 2A ligands (warfarin, bilirubin, prostaglandin, furosemide, and coumarin) to the HSA mutants produced in this study in order to determine what role arginine 218 may play in modulating the binding of other ligands to subdomain 2A.

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