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^{19}F NMR Studies of Tryptophan/Serum Albumin Binding

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Abstract—^{19}F NMR provides direct measures of the Trp binding avidity of 'fatty acid free' bovine serum albumin when D- and L-6-fluorotryptophan are used as the probes. Both a high and low affinity binding site are present. The addition of octanoate either displaces the ligand from both sites or greatly decreases the affinity such that little binding occurs at 2 mM levels. In the case of L-6-fluorotryptophan separate signals are observed for the high and low affinity binding sites and titrations with competing ligands can be used to establish the relative affinities of ligands at the high affinity site. Binding at this site appears to be hydrophobic and shape specific with L-Phe being a very poor ligand (K_D[L-Phe]/K_D[L-Trp] = 800) while both GHKαNal and GHKW displace L-6-fluorotryptophan from this site. In tripeptides of the general formula GHK[εNH$(CH_2)_n$(CO)W], affinity increases with tether length and binding at the low affinity site is restored. This NMR assay appears well-suited for the discovery of selective binding agents in this and other biorecognition phenomena.

Introduction

Serum albumin is the most abundant protein in blood plasma; its two major roles are maintaining osmotic pressure and depositing and transporting a variety of endogenous and exogenous compounds. Medium- and long-chain fatty acids, bilirubin, and tryptophan (Trp) are a few examples of the endogenous substances bound to this protein. A large number of drugs (notably, warfarin and ibuprofen) also bind to albumin. The interaction between the ligands and serum albumin results in an increased carrying capacity of blood plasma for otherwise insoluble molecules, a toxicity decrease for some toxic ligands, and provides sequestration mechanisms that protect unstable species and allow for their slow release.[1–5] The Trp/albumin interaction is among the more studied of these ligand binding phenomena due to its medicinal significance. The concentration of L-Trp in blood determines its concentration in the brain, which in turn determines the rate of synthesis of the neurotransmitter serotonin (5-hydroxytryptamine). Anomalous binding of Trp in serum albumin has been associated with certain types of schizophrenia[6] and rheumatoid arthritis.[7]

Much of the literature on the binding of Trp to bovine and human serum albumin (BSA, HSA) suggests that Trp interacts stereospecifically with the protein. One study reports that the binding constant for L-Trp is approximately 2×10^5 M^{-1}, whereas that for the D enantiomer is 1×10^4 M^{-1}.[8] Other studies have suggested a 100-fold difference[3] or have assumed that the binding of the D-isomer is sufficiently less avid so that it can be ignored.[9] In an early ^{19}F NMR study of L-5-fluorotryptophan (L-5-F-Trp) binding to HSA, Gerig and Klinkenborg[10] concluded that at least two distinct binding sites exist for Trp on fatty-acid-free HSA at pH 7.4: a high affinity site with $k_{off} < 10$ s^{-1}, and one or more low affinity binding site(s). Jenkins and Lauffer[9] also used ^{19}F NMR to study ligand binding to defatted HSA. The racemic forms of both 6-F-Trp and 5-F-Trp were examined but the study focused on the 5-F derivative since it displayed a larger binding-induced shift. The 'tight binding' site was found to bind only one equivalent of 5-F-Trp and the upper limit of the dissociation constant was set at 2.5×10^{-5} M, assuming only weak nonspecific binding of the D-isomer. Ibuprofen was found to be the most powerful displacer of 5-F-Trp from this site, while palmitate was found to be a weak displacer. Chloride ion (which was present in the solutions used by Gerig and Klinkenborg) also 'displaces' 5-F-Trp.[9] In order to further characterize the albumin binding sites for Trp, and to search for selective binding agents, we have employed BSA with L- and D-6-fluorotryptophan (6-F-Trp) as probes in ^{1}H NMR relaxation studies and displacement titrations followed by ^{19}F NMR. Our studies confirm the presence of at

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least two binding sites and extend the method to provide relative affinities and establish that agents can be designed which select between the alternate binding sites for Trp. The results also rationalize the previous failures to derive a single bound-state conformation for Trp using transferred-NOE methods.[11]

Results and Discussion

^{19}F NMR is well-suited for studies of ligand/protein binding. For high-affinity sites (which are not in rapid exchange with free ligand on the NMR shift timescale), the ^{19}F chemical shift should be different for each site and the D- and L-Trp complexes are diastereomeric and thus could also display different shifts. In addition, the signal linewidth should reflect the correlation time of albumin tumbling and these linewidth effects can, in analogy to transferred NOEs,[12–14] be transferred to the signal that represents the average of the free and loosely bound species. It is, however, difficult to extract binding constants from linewidths and we rely, for affinity measures, exclusively on integral ratios that reflect the fraction bound; specifically, the integral ratio (**R**) of the intensity for a specific bound signal to the total of all ^{19}F signals.

$$\mathbf{R} = \frac{\text{specific-bound-}^{19}\text{F}}{\text{total } ^{19}\text{F}}$$

The change in this ratio with ligand/BSA ratio can provide the stoichiometry of the specific binding site and changes during competition experiments can provide relative affinities of the competing ligands.

The linewidth of exchange-averaged peaks, as the width at half-height (hw) in the present study, can provide qualitative data regarding the bound fraction within the exchange average signal but can, in certain exchange regimes, also reflect exchange with tight binding sites. In the absence of a specific binding signal, a quantitative measure of the relative affinities cannot be derived since it is difficult to assess the separate line broadening effects of 'specific' versus 'loose' binding site occupancy. The hw measurement also depends on experimental parameters (shimming, other relaxation sources, etc.) and these varied during the course of the study. As a result, only changes during a titration at constant protein concentration (not the absolute hw values) are used in the present study.

Affinity and enantioselectivity of 6-fluorotryptophan binding to BSA

In order to confirm binding to BSA we examined the effect of BSA/ligand ratio upon the selective and non-selective relaxation rates of the aromatic protons of 6-F-Trp (data not shown). As expected for fast equilibrium binding, $^{s}R_1$ was sensitive to the ratio with both enantiomers displaying similarly enhanced relaxation at high BSA/ligand ratios. With only a 6-fold excess of BSA, the L-enantiomer display faster relaxation suggesting higher affinity (a larger bound fraction in equilibrium with the free species) but it was clear that this method would neither serve to distinguish the number of sites nor define the affinities and enantioselectivities of binding since both enantiomers were significantly bound at NMR concentrations.

In contrast, fluorine NMR allowed us to examine the interactions at stoichiometries that distinguish the enantiomers. Key spectra from the titrations are shown in Figure 1. For the L-enantiomer, the ^{19}F probe is distributed between a broad peak ($\delta = -42.7$ ppm, $hw = 108.5 \pm 2.0$ Hz, independent of the ligand/BSA ratio) and an exchange-averaged peak at essentially the same chemical shift as observed for 6-F-Trp in the absence of BSA ($\delta = -46.15$ ppm). This upfield peak becomes increasingly sharp as the free ligand fraction increases. In the absence of BSA (or with excess L-Trp added), the upfield peak is observed with hw measurements typically near 46 Hz (sometimes as low as 30 Hz when shimming and sample preparation was optimal). In contrast, D-6-F-Trp displayed only the upfield peak, no tightly-bound state with a distinct shift was observed. More specific analysis of these two titration experiments appears below.

In the case of the D-enantiomer, no binding constants can be derived from the titration data. The decreasing linewidth of the signal as the ligand/BSA ratio is increased, and the more dramatic sharpening that occurs upon addition of excess L-Trp confirm that binding is occurring. If we take the hw observed for the downfield peak seen with L-6-F-Trp as the expectation value for 100% bound, the linewidths observed with D-6-F-Trp suggest that more than one molecule is bound at the 6 mM point of the titration. The absence of ^{19}F chemical shift changes during the titration implies that any bound state(s) of the D-enantiomer have intrinsic chemical shifts that are not very different from the free solution state.

In the case of L-6-F-Trp, we took particular care to obtain spectra with useful signal/noise ratios even at the lowest loading of the ligand in order to attempt to define the binding constant. At the lowest loading (0.45 molar equivalents of ligand), only the downfield signal due to the bound state at the higher affinity binding site is clearly evident. As a greater (but still less than stoichiometric) quantity of ^{19}F probe is added, another broad peak ($hw = 103$ Hz) appears. We view this as evidence for (at least) two Trp-binding sites on BSA. At this loading, both ^{19}F peaks display comparable linewidths (corresponding to that expected for the correlation time of albumin) while the **R** value reflects the competition of two or more BSA sites for a limited quantity of the common ligand. Unfortunately the less populated site is nearly shift coincident with the signal for the free ligand. As more of the ^{19}F probe is added, an exchange-averaged signal of decreasing width appears at this shift, due to the increasing contribution of free ligand. The absolute intensity of the high-affinity bound state signal at -42.7 ppm does not increase as

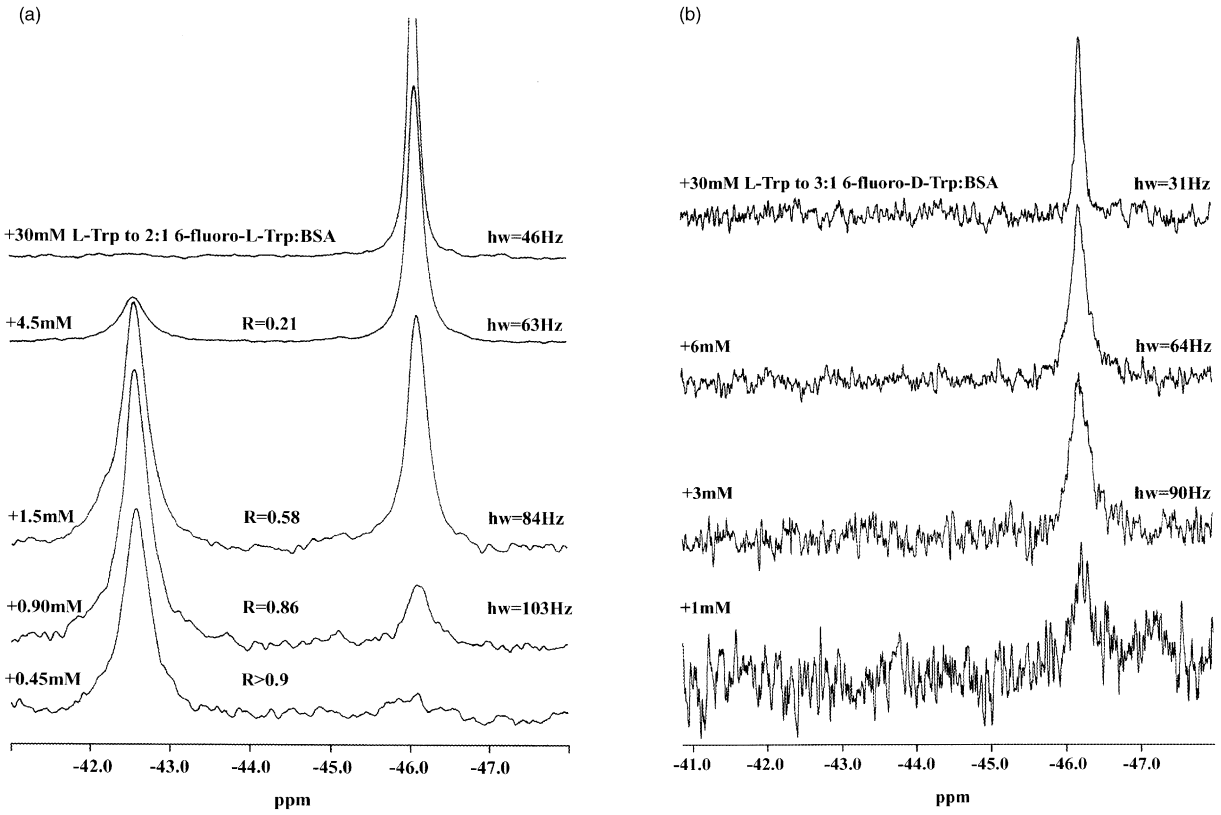

Figure 1. ^{19}F-monitoring of the addition of 6-fluorotryptophan to 1 mM BSA in D_2O solution at pH 7.2 and 302K: (a) the addition of 0.45 to 4.5 molar equivalents of L-6-F-Trp; (b) the addition of 1–6 molar equivalents of D-6-F-Trp. In each case, the effect of the addition of excess non-fluorinated L-Trp is also shown in the top trace. In panel B, the lowest concentration trace provides no information since the signal to ratio is too low for the extraction of linewidths; also, the −47.2 ppm feature was not reproducible.

the L-6-F-Trp/BSA ratio is increased to 2, 3 4.5 and 6 (data not shown). At all of these ligand/BSA ratios, the **R** value indicates, within experimental error, a 0.85 mM concentration of this bound state—the deviation from 1.0 mM (the total BSA concentration) is likely due to residual binding species in the albumin preparation.

With the concentration of the high affinity site established it is possible to use the integral ratio (**R**) and stoichiometry to estimate the binding affinity for L-6-F-Trp from the first few points of the titration appearing as Figure 1a.

$$K_D = \frac{[BSA]_T\{0.85 - (\boldsymbol{R}[L]_T)\} \times \{[L]_T - \boldsymbol{R}[L]_T - [L]_{nsb}\}}{\boldsymbol{R}[L]_T} = \frac{[P]\times[L]}{[L\cdot P]}$$

In adopting this expression, we assume that albumin with ligand associated only at the low affinity site can still bind L-6-F-Trp at the high affinity site. However the binding of the *ligand* at non-specific sites does reduce the free ligand concentration. The correction for this, the concentration of non-specifically-bound ligand ($[L]_{nsb}$), is the weakest link in the derivation of dissociation constants. Without the correction, the K_D-values for L-6-F-Trp fall in the range $3.9–6\times10^{-5}$ M. With the correction, estimated from the hw of the exchange-averaged signal, the range is $0.7–4\times10^{-5}$ M. Given the distribution between the high and low affinity sites observed at low loadings, the dissociation constant at the low affinity site is greater by at least a factor of 20.

Tryptophan/BSA binding

Both enantiomers of Trp compete with L-6-F-Trp for the high and low affinity binding sites on BSA (Fig. 2). Increasing the amount of unfluorinated Trp diminishes the amount of L-6-F-Trp that is bound at the high affinity site and narrows the exchange-averaged signal. When a large amount of Trp is added to the L-6-F-Trp/BSA solution, the bound state signal completely disappears and the exchange-averaged signal is restored to linewidths characteristic of the free solution state in this medium (Fig. 1a). The two sites are designated as 'specific' and 'low-affinity' henceforth. From the titration studies, the binding of Trp to BSA at the specific site was estimated to be 18 times stronger than that of the D enantiomer to the same site, which is essentially the same as the estimate from gel filtration.[8] The dissociation constant of the specific site L-Trp/BSA complex can be bracketed in the range $0.7–4\times10^{-6}$ M. All further binding data are expressed as relative affinities with L-Trp set as 100 since the relative affinities are more precise than the absolute values of the derived dissociation constants.

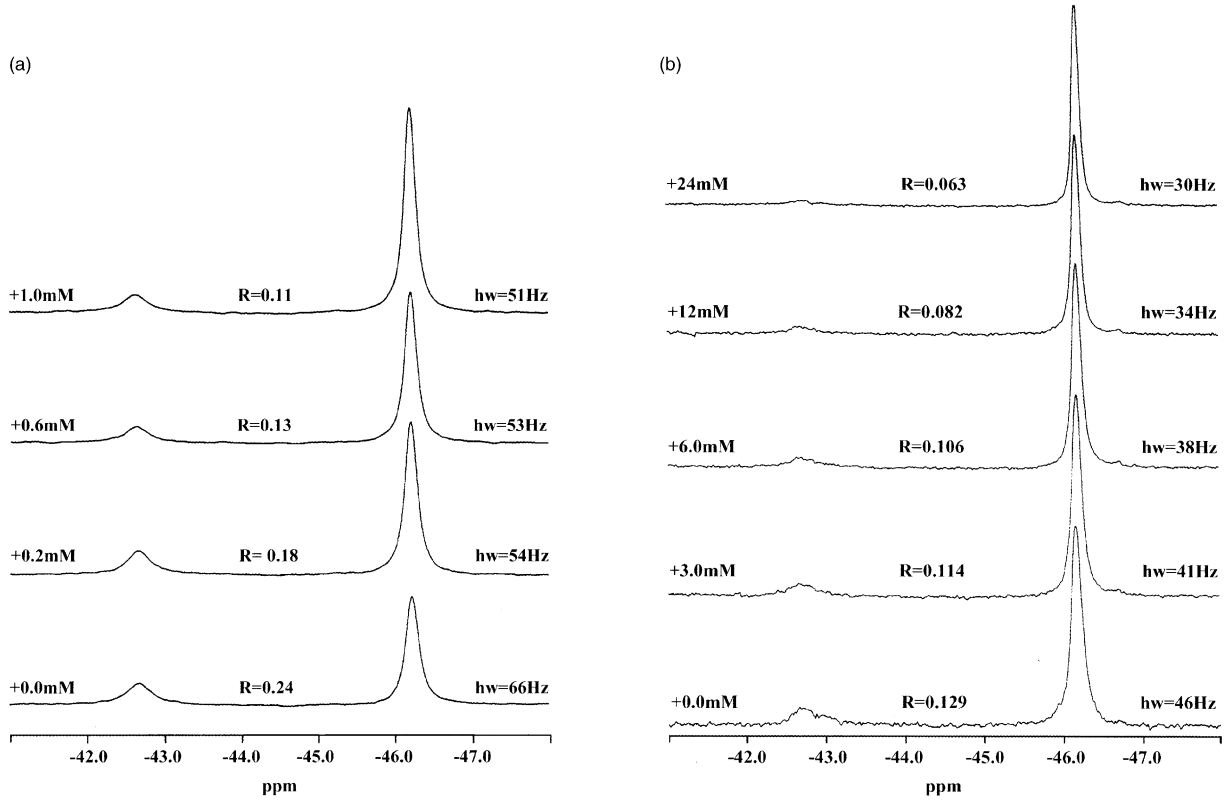

Figure 2. Equilibrium displacement of L-6-F-Trp bound to BSA (D_2O solution at pH 7.2 and 302K) by L-Trp (a) and D-Trp (b). (a) The L-Trp series starting point is 3:1 L-6-F-Trp/BSA solution, [BSA] = 1 mM. (b) The D-Trp series starting point is 4:1 DL-6-F-Trp/BSA solution, [BSA] = 1 mM. In both cases, the signal at −42.7 ppm is due exclusively to L-6-F-Trp binding at the 'specific' site.

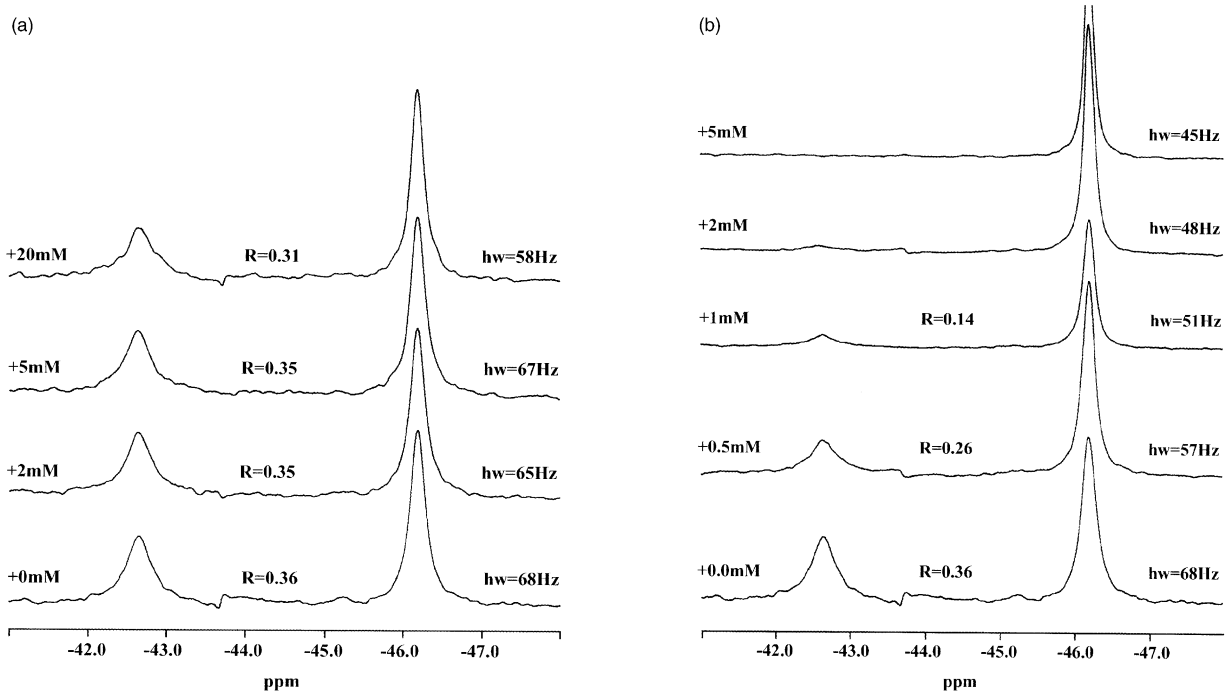

Figure 3. The effect of addition of increasing amounts of L-phenylalanine (panel a) and octanoic acid (panel b) to 2:1 D-6-F-Trp/BSA (D_2O solution at pH 7.2 and 302K, [BSA] = 1 mM).

The selectivity for the amino acid sidechain is even greater than the enantioselectivity. Figure 3a shows the very modest level of competition seen in a titration with L-phenylalanine. Both the 'specific' and 'low-affinity' sites show remarkable selectivity for the indole ring sidechain. For the 'specific' site the following relative affinities can be derived: Trp (100), L-6-F-Trp (9.7), D-Trp (5.5), and L-Phe (0.12).

The ^{19}F NMR assay was also used to confirm the conclusion of King and Spencer[15] that medium-chain fatty acids displace Trp from serum albumin. The addition of as little as 2 equivalents of octanoic acid eliminates the signal due to the specifically bound ^{19}F-tag and reduces the hw of the exchange-averaged signal to those observed with excess Trp (Fig. 3b). The data does not allow an unambiguous distinction between competitive binding and octanoate-induced changes in affinity at other sites. The loss of ^{19}F-tag binding, as reflected in both the −42.7 ppm intensity and the hw of the exchange-averaged signal, at low octanoate levels suggests a non-competitive 'displacement' mechanism. The experiment does provide clear evidence that fatty acid free albumin must be used for these studies.

Selective binding of tryptophan containing peptides

In contrast to free L-Trp binding to BSA, Trp placed in tetrapeptide Gly-His-Lys-Trp (GHKW), displaces L-6-F-Trp from the 'specific' binding site (Fig. 4a), but does not reduce the linewidth of the exchange-averaged signal (due to low-affinity bound and free states). GHKW is a selective ligand that binds only to the specific site of BSA, but with a reduced binding constant (~50 times lower affinity than L-Trp). In order to establish that this is competitive binding, we prepared GHK-6-F-Trp for binding studies. The ^{19}F-signal of the tetrapeptide incorporating L-6-F-Trp appears at −47.0 ppm (hw = 93 Hz) and shifts to −46.8 ppm with a diminished linewidth as additional tetrapeptide and/or unlabeled Trp is added (data not shown). Other NMR studies have established that with the reduced affinity resulting from incorporating 6-F-Trp into the tetrapeptide, exchange with both Trp sites is rapid on the NMR timescale.

Displacement studies with GHK-Xaa species

The naphthalene ring resembles, and is an excellent replacement for, the indole ring of Trp; GHKαNal displaces L-6-F-Trp even better than GHKW and the displacement is extended to the low affinity site (Fig. 4b). Control experiments (data not shown) with tripeptides GHK and HGK established that this portion does not provide affinity. GHKVFV shows essentially no displacement of L-6-F-Trp from the specific site but does produce some reduction in the hw of the exchange-averaged L-6-F-Trp signal (data not shown). Both sites require hydrophobic groups.

When a flexible tether of variable length is inserted between lysine and Trp in GHKW, the resulting species act increasingly like free Trp. These species, GHK[εNH$(CH_2)_n$(CO)W], displace L-6-F-Trp from the specific site and narrow the exchange-averaged signal,

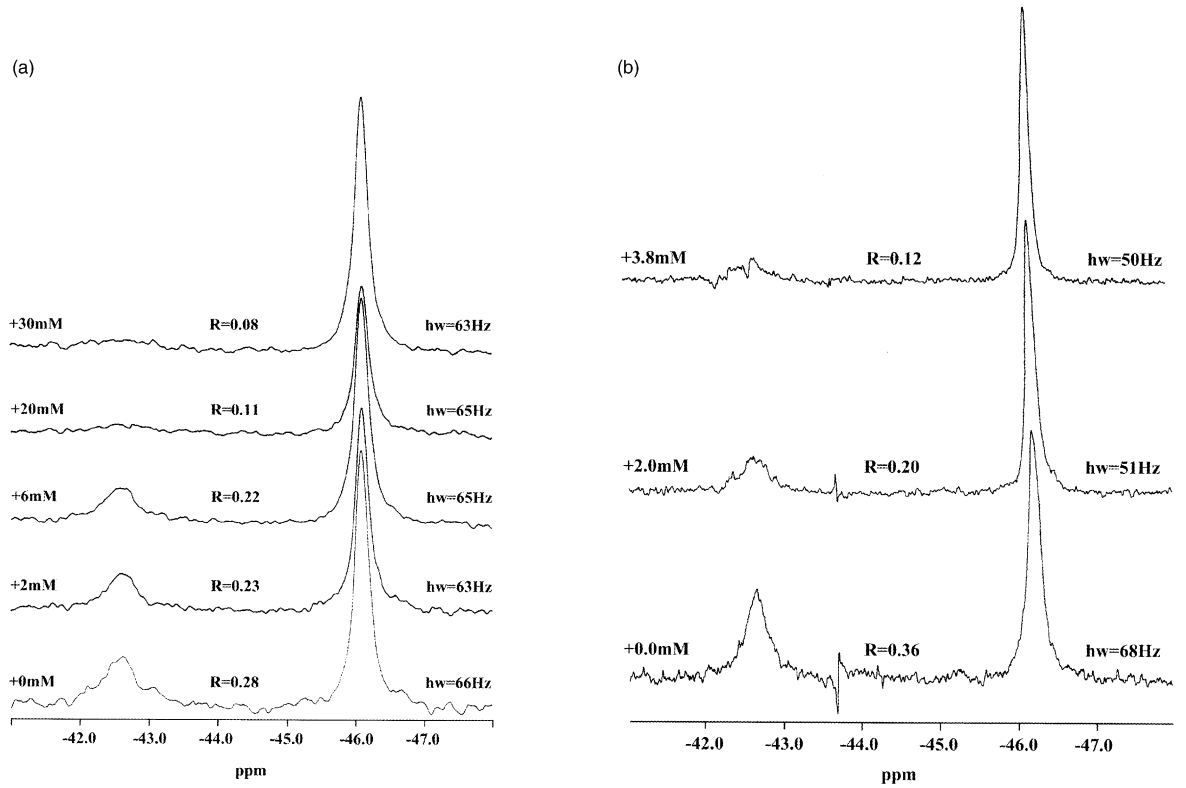

Figure 4. Displacement of L-6-F-Trp from BSA by GHKW (panel a) and GHKαNal (panel b). In both cases, the BSA concentration is 1 mM; the initial L-6-F-Trp/BSA ratios were 3:1 (panel a) and 2:1 (panel b) with the titrations performed at pH 7.2 and 302 K.

which indicates competition at both sites (Fig. 5) and a loss of selectivity.

Conclusions

The use of F-tagged probes and ^{19}F NMR provides a very sensitive assay for biorecognition phenomena. In the present case, it has served to establish both the enantio- and sidechain-specificity of the serum albumin sites that bind Trp. Based on stoichiometry calculations, the K_D for L-Trp may be as low as 7×10^7 M and is certainly less than 6×10^6 M — more avid binding than reported in any other study. The relative affinities of a number of amino acids and peptides were assessed (Table 1).

We have not addressed the previously determined[3] requirement of a carboxyl function for high affinity binding, but this study does indicate that there is a significant hydrophobic component and that the shape of the aromatic moiety is important. The naphthalene ring is the best substitute for the indole ring of Trp; in the tetrapeptide context, the affinity increases by a factor of seven when αNal replaces Trp.

While the relative affinities at the lower affinity site could not be quantitated, the data suggests that binding is less enantioselective. Even in the limited series of analogues examined herein, significant selectivity between the sites has been observed; the two sites have different requisites for binding. The results suggest that it will be possible to develop highly selective agents for the specific and low affinity sites. Further, we anticipate that F-tagging of drug candidates (and other protein ligands) will prove to be a very powerful technique in the study of biorecognition phenomena and in structure/binding-based drug design.

Regarding prior attempts to derive the albumin-bound-state geometry of L-Trp using transferred NOEs, even though D-Trp was employed as a control and displayed different NOE ratios,[11] it appears likely that the data reflects geometries at the low affinity site, particularly

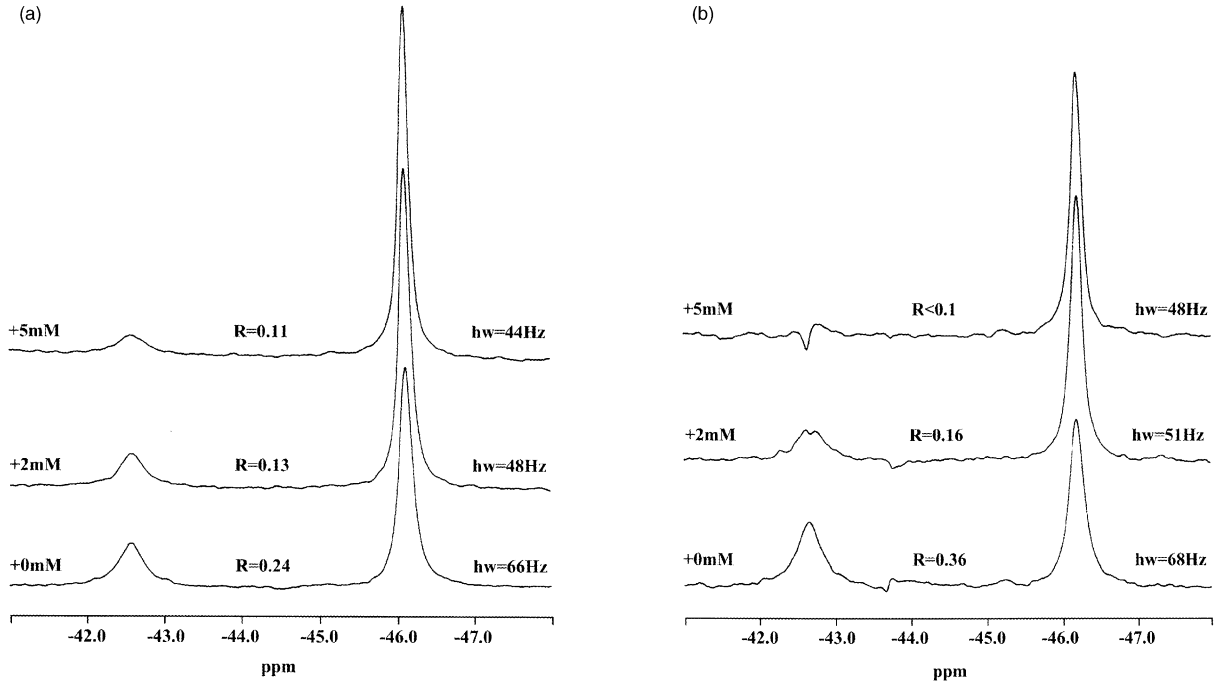

Figure 5. The effect of the addition of 2 and 5 equivalents of GHK[εNH$(CH_2)_n$(CO)W] to L-6-F-Trp/BSA complex at pH 7.2 and 302 K, [BSA] = 1 mM. (a) $n = 3$, with 3 equiv L-6-F-Trp (b) $n = 5$, with 2 equiv L-6-F-Trp.

Table 1. Relative BSA affinities measured by L-6-F-Trp displacement

Ligand	Specific site relative affinity	Low-affinity binding
L-Trp	100	Avid
GHK[εNH$(CH_2)_5$(CO)W]	20	Avid
GHKαNal	14	Avid
GHK[εNH$(CH_2)_3$(CO)W]	10.2	Modest
L-6-F-Trp	9.7	Avid
D-Trp	5.5	Avid
GHKW	2.1	Weak
GHK-L-6-F-Trp	<1	n.d.
GHKVFV	0.97	Modest
HGK	<0.35	None
L-Phe	0.12	Weak

for the L-Trp. In order to determine the geometry of the specifically-bound species, BSA will need to be in significant excess and that will require universal ^{13}C-labeling of the Trp in order to distinguish the ligand signals.

Experimental

Phosphate buffer (62.5 mM, pH 7.2, throughout) was prepared by dissolving 26.7 mg K_2HPO_4 and 13.2 mg of KH_2PO_4 in 4 mL of 99.96% D_2O, lyophilizing and reconstituting with 99.96% D_2O. Titrant solutions of 6-F-Trp samples were prepared by dissolving 1.11 mg of 6-F-Trp in 0.5 mL of 99.96% D_2O, which was removed by lyophilyzation. The samples were then dissolved in 0.5 mL of 50 mM phosphate D_2O buffer at pH 7.2. The following 6-F-Trp samples were employed: DL- (Sigma), the resolved enantiomers thereof (obtained by conversion to the ethyl ester followed by treatment with α-chymotrypsin at pH 5, separation of the ester and acid, etc.), and commercial L-6-F-Trp (Fluka). The resolved samples were assayed by circular dichroism: L-isomer ($[\theta]_{223nm} = +17{,}000^\circ \cdot cm^2$ $dmol^{-1}$), D-isomer ($[\theta]_{223nm} = -20{,}400^\circ \cdot cm^2 \cdot dmol^{-1}$). L-Trp, D-Trp, L-Phe and octanoic acid were 98 + % pure commercial materials. Other 10 mM peptide (Gly-His-Lys-Trp, etc.) samples were prepared similarly in D_2O, not buffer. All of the GHK-Xaa peptides were gifts from Procyte Corp. (Seattle, WA, USA); the sequences and purities were confirmed by 2D ^1H NMR spectroscopy (as DMSO-d_6 solutions) at 500 MHz. Commercial 'essentially fatty acid free' BSA (Sigma A-6003, Cohn fractionation followed by defatting according to Chen[16]), as obtained from the supplier, was employed throughout. BSA stock solutions were prepared by dissolving 201 mg of the protein in 3.0 mL of 62.5 mM phosphate buffered D_2O. The BSA/ligand solutions were prepared by adding a measured amount of small molecule solution(s) to a portion of the BSA solution to obtain the desired protein/ligand ratio. For titrations of non-fluorine containing species to 6-F-Trp/BSA solution, the non-fluorine containing species were dissolved in 99.96% D_2O rather than buffer. Throughout the final buffer concentrations in the NMR samples was ca. 50 mM with an albumin concentration of 0.85–0.95 mM. To simplify the account and figure legends, [BSA] is reported as 1 mM with all ligand amounts adjusted to this basis.

The NMR spectra were performed on a Bruker AF300 and AM300 spectrometers with a ^{19}F operating frequency of 282.3 MHz. Samples of each 6-F-Trp isomer, with and without added BSA were also spiked with trifluoroacetate (ca. 1 mM, final) for chemical shift calibration; TFA was set to 0.0 ppm, rather than its standard value of 77 ppm relative to $CFCl_3$. All ^{19}F spectra were recorded without proton decoupling at digital resolutions ranging from 1.0 Hz/point to 2.1 Hz/point. The actual resolution was on the order 6 Hz, proton/fluorine splitting were not well-resolved prior to line broadening. The 'number of scans' was chosen based on the ^{19}F concentration of the samples. The preparative delay (always > 2 s) was kept constant in each titration to insure reproducible integrals. The ^{19}F NMR data was processed on the Aspect 3000 computers connected to the spectrometers. The signal/noise (S/N) ratio was enhanced by apodizing (LB = 5 Hz) the time domain signals of ^{19}F prior to Fourier transformation.

Acknowledgements

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