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Analysis of Protein/Ligand Interactions with NMR Diffusion Measurements: The Importance of Eliminating the Protein Background

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Pulsed-field gradient nuclear magnetic resonance (PFG-NMR) is a well-established method for the determination of translational diffusion coefficients. Recently, this method has found applicability in the combinatorial arena with the introduction of affinity NMR for characterizing protein/ligand interactions. Although affinity NMR has been reported to be an effective method for the identification of active compounds in a complex mixture, there are limitations of this method. We have developed a simple mathematical model to predict optimum concentration ratios of the ligand and protein in order to observe maximum changes in the ligand diffusion coefficient upon protein binding. The ligand/protein systems of L-tryptophan and ibuprofen binding to human serum albumin were chosen to demonstrate the usefulness of this model. However, even when the conditions of the mathematical model are satisfied, the spectral background arising from the protein in proton-detected experiments can be problematic. To this end, we have employed spectral subtraction of the protein spectrum to yield ligand diffusion coefficients that are in agreement with those predicted by simulation.

Key Words: **PFG-NMR; affinity NMR; diffusion; ligand binding; albumin.**

INTRODUCTION

Protein functions such as the immune response, enzyme catalysis, ion transport, and gene transcription and translation, are largely mediated by ligand recognition and binding. There are many methods available for probing protein/ligand interactions including equilibrium dialysis, ultrafiltration, size-exclusion chromatography, capillary electrophoresis, and fluorescence spectroscopy (*1*). The disadvantages of these methods include the necessity for some kind of separation during the analysis, which can perturb the binding equilibrium, and the need for fluorescent probes or derivatization for labeling purposes. This paper focuses on the use of pulsed-field gradient nuclear magnetic resonance (PFG-NMR), a method that has been employed extensively for mixture analysis (*2–6*). Due to the non-invasive nature of NMR, it is possible to probe these interactions without disturbing the binding equilibrium. Furthermore, all organic ligands are amenable to analysis by NMR, potentially eliminating the need for derivatization.

There are several NMR parameters that are commonly exploited in the investigation of protein–ligand binding including chemical shift, linewidth and relaxation parameters, NOE, and diffusion measurements. For example, by comparing the changes in protein chemical shifts upon ligand binding, it is possible both to detect binding and to locate the binding site and orientation of protein-bound ligands (*7*). Chemical shift analysis has also been used successfully to identify ligands that bind weakly to a labeled protein in an attempt to produce a high-affinity ligand (*8, 9*). Changes in relaxation parameters and the observation of NOEs, particularly transferred NOEs in the case of protein/ligand interactions, are not usually employed for the quantitation of ligand binding but are extremely useful for elucidating the bound conformation of the ligand (*10, 11*). PFG-NMR measurements of translational diffusion coefficients (*12, 13*) have been previously used as an indication of binding (*6, 14, 15*) or aggregation events (*16–18*) based on changes in hydrodynamic radius resulting from these associative processes. An interesting application of PFG-NMR, affinity NMR, has been recently reported as a method for detecting the ligands in a complex mixture that bind to a target protein (*6, 19*).

Affinity NMR is a relatively new approach in which bound ligands are identified based on the change in the observed ligand diffusion coefficient resulting from protein binding (*20, 21*). Affinity NMR experiments are typically performed using solutions with a constant protein concentration to which a mixture of ligands all at similar concentrations, such that the ligand concentrations are greater than that of the protein, is added. This technique permits the identification of active compounds in a combinatorial mixture without a physical separation or the use of labels. In addition, "false positives" that can arise from additive effects of all ligands in a mixture instead of from just one specific ligand can be eliminated with this method (*20*). Unlike chemical shift measurements, the strategy of affinity NMR relies on changes in the diffusion coefficient of the ligand that result from binding to the much larger and more slowly

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diffusing protein. Having selected the ligands of interest based on their diffusion coefficient, the binding ligand can then be structurally characterized while still present in the mixture by combining a diffusion pulse sequence with a two-dimensional NMR experiment such as TOCSY (*5, 22*). This approach works well for relatively simple systems; however, there are inherent limitations of this method that can affect the feasibility of the experiment.

The main criterion for affinity NMR is that there must be significant differences in the observed diffusion coefficients of binding and nonbinding ligands in order for them to be differentiated. This difference in the free and bound ligand diffusion coefficients defines the effective analytical dynamic range of the measurement. Systems amenable to this approach are those in which the binding kinetics are in the fast exchange regime on the NMR chemical shift and diffusion time scales. In the fast exchange limit, the observed ligand diffusion coefficient is a weighted average of free and bound states. Because the bound ligand usually has unfavorable relaxation properties, it is desirable to perform the experiments under conditions where some free ligand is present. However, because the observed ligand diffusion coefficient is a weighted average, a large excess of the free ligand can effectively reduce the dynamic range of the affinity NMR measurement. Therefore the protein/ligand ratio must be selected carefully to produce the best results. We present here a simple mathematical model that predicts the optimum range of protein/ligand ratios for specific binding interactions (i.e., a single binding site) over a wide range of binding affinities.

Another limitation of these measurements is the spectral background resulting from the resonances of the protein that can overwhelm the ligand signals in proton-detected experiments. Even when the ligand molar concentration is significantly greater than that of the protein, the protein background can lead to false or misleading data and result in invalid conclusions. We have employed spectral subtraction to effectively eliminate the error that the protein background signal contributes to the observed ligand diffusion coefficient. Ligand diffusion coefficients measured with and without protein background subtraction are compared with the values predicted by the mathematical model.

The protein/ligand systems selected for this study are L-tryptophan and ibuprofen, both of which bind to the protein human serum albumin (HSA). L-tryptophan binds to HSA at a single binding site with moderate affinity ($K_d = 160\,\mu M$), while ibuprofen has a single high affinity site as well as several lower affinity sites (*23–25*). The binding of HSA to various ligands, including L-tryptophan and ibuprofen, has been previously characterized (*26, 27*). HSA is the most abundant blood protein in humans and serves as a transport protein for many endogenous and exogenous compounds (*28*). Consequently, it binds many ligands, of which L-tryptophan and ibuprofen are just a few. This protein is well suited for our study because the concentrations that are needed for the NMR experiments are biologically relevant (*26*) and because it can be obtained in large quantities inexpensively and in relatively pure form.

RESULTS AND DISCUSSION

For protein/ligand binding equilibria in fast exchange on the NMR diffusion time scale the translational diffusion coefficient observed for the ligand, D_o, is a weighted average that can be expressed by the equation

$$D_o = F_f D_f + F_b D_b \qquad [1]$$

where F_f is the fractional concentration of the free ligand, D_f is the diffusion coefficient of the free ligand, F_b is the fraction of the total ligand that is bound to the protein, and D_b is the diffusion coefficient of the bound ligand (assumed to be the diffusion coefficient of the free protein). Assuming a 1 : 1 protein : ligand binding equilibrium, the normal expressions for the equilibrium can be written

$$LP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} L + P \quad K_d = \left[\frac{[L][P]}{[LP]}\right], \qquad [2]$$

where K_d is the dissociation constant of the protein/ligand complex [LP], [L] is the concentration of free ligand, and [P] is the concentration of free protein. By solving Eq. [1] for the fractional concentrations of free and bound ligand in terms of diffusion coefficients, Eq. [2] can be manipulated so that it is expressed entirely in terms of parameters that are known or can be measured:

$$K_d = P_{tot}\left(\frac{D_b - D_o}{D_o - D_f}\right) + L_{tot}\left(\frac{D_o - D_b}{D_b - D_f}\right), \qquad [3]$$

where P_{tot} is the total protein concentration and L_{tot} is the total ligand concentration.

The calculation of the dissociation constant in Eq. [3] assumes a 1 : 1 binding equilibrium between the ligand and protein, fast exchange on the NMR diffusion time scale, and that the NMR resonances of the ligand are not excessively broadened upon binding. As shown graphically in Fig. 1 it is possible to manipulate Eq. [2] to simulate the expected change in the ligand diffusion coefficient ($\Delta D = D_f - D_o$) as a function of ligand : protein molar ratio over a wide range of K_d values. Figure 1 was generated by calculating values of ΔD in an Excel spreadsheet for ligand : protein molar ratios ranging from 0.1 : 1 to 500 : 1 for each K_d curve. Regardless of the value of K_d, the curves all converge to zero as the ligand concentration becomes large relative to that of the protein and the observed diffusion coefficient approaches that of the free ligand. However, the behavior of ΔD at low ligand : protein molar ratios varies with the value of K_d. For high-affinity binding sites (low K_d values) and low ligand:protein molar ratios, essentially all the ligand is bound to the protein and the observed diffusion coefficient will be that of the protein. As the magnitude of K_d increases, the equilibrium favors the free ligand and even at low ligand : protein molar ratios the observed ligand diffusion coefficient reflects a substantial fraction of free ligand. Figure 1 indicates that for intermediate

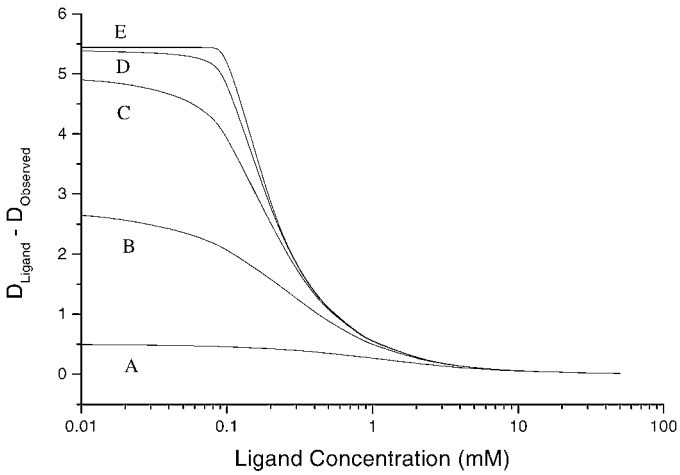


FIG. 1. Assuming a HSA protein concentration of 0.1 mM, HSA diffusion coefficient of 0.633×10^{-10} m^2/s, and a free L-tryptophan ligand diffusion coefficient of 6.07×10^{-10} m^2/s, corrected for the viscosity of the HSA solution, the change in diffusion coefficient of the ligand ($D_{\text{free}} - D_{\text{o}}$) as a function of ligand concentration is simulated for various dissociation constants. $K_{\text{d}} = 1000\,\mu$M (A), $K_{\text{d}} = 100\,\mu$M (B), $K_{\text{d}} = 10\,\mu$M (C), $K_{\text{d}} = 1\,\mu$M (D), $K_{\text{d}} = 0.001\,\mu$M (E). The curves displayed in this figure were obtained by solving equation 3 for the observed ligand diffusion coefficient, D_{o}, and calculating the difference in the ligand diffusion coefficient, $D_{\text{free}} - D_{\text{o}}$, as a function of ligand concentration and the dissociation constant, K_{d}.

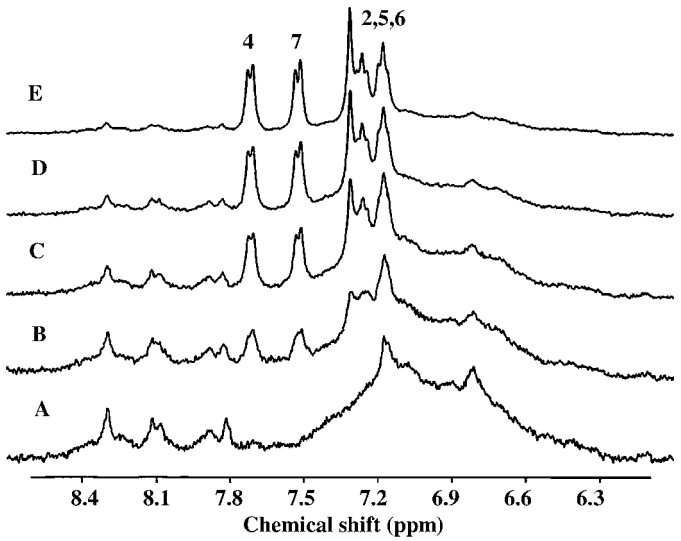


FIG. 2. Aromatic region of L-tryptophan : HSA mixtures at various molar ratios: 3 : 1 (B), 5 : 1 (C), 7 : 1 (D), 10 : 1 (E). The same aromatic region of free HSA in the absence of L-tryptophan is shown in (A).

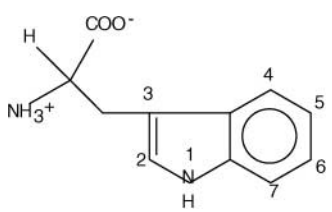
ligand : protein concentration ratios (i.e., 1–5), the value of ΔD depends on both the concentration ratio and K_{d} and therefore should provide information about the relative affinity of different ligands for a protein binding site.

Diffusion Measurements of L-Tryptophan Binding to HSA

Because HSA has a single L-tryptophan binding site, this is a good model system to test our ability to predict ligand diffusion coefficients as a function of the ligand : protein ratio. L-tryptophan (structure in Scheme 1) is not a very common amino acid in proteins and the resonances of the indole ring are shifted slightly downfield from those of tyrosine and phenylalanine residues of HSA. Therefore, an additional advantage of this ligand is that some of its aromatic NMR resonances are reasonably well resolved from those of HSA, even at the relatively low magnetic field used in this study.

At the lowest ligand : protein ratio studied (1 : 1), the tryptophan resonances were not observable due to spectral overlap from the HSA resonances. As shown in Fig. 2, at a ligand : protein ratio of 3 : 1 the aromatic resonances of L-tryptophan could be observed, but still suffered from overlap with the resonances of the protein. In addition, these tryptophan resonances are noticeably broadened as a result of binding to HSA. As the ligand : protein molar ratio was increased the signal-to-noise ratio of the ligand resonances naturally increased as well. In addition, the tryptophan aromatic resonances became sharper and the J-coupling resolved. However, even with a 10-fold excess of ligand, one can discern from simple visual inspection of the spectrum that protein background could contribute significantly to the integrated intensity of ligand resonances. As shown in Table 1, the ligand diffusion coefficients measured for these solutions are all lower than those predicted by our simulation due to contributions to the integrated L-tryptophan resonance intensity from underlying HSA resonances. Clearly the protein background has the potential to produce misleading results in the analysis of NMR diffusion data.

In an endeavor to reduce problems arising from the protein background, it seems reasonable to use large ligand : protein ratios so that the contribution from the protein background is minimized. However, for specific binding interactions the use of relatively large ligand concentrations places one outside the optimum concentration regime for detecting changes in the ligand diffusion coefficient. Relaxation editing can also be used to reduce the background due to the protein magnetization by incorporating a spin echo or CPMG pulse train into the diffusion pulse sequence (*29, 30*). However, this method may also greatly reduce the intensity of the ligand resonances, which are broadened as a result of protein binding. Alternatively, spin-labeling of ligands has been used to eliminate protein background. Gonnella *et al.* addressed this problem utilizing isotope-filtered affinity NMR in which the proton NMR signals arising from the protein and nonbinding ligands are eliminated using ^{13}C isotope editing



SCHEME 1

TABLE 1
Effect of HSA Binding on the L-Tryptophan Diffusion Coefficients ($\times 10^{-10}$ m^2/s)

Ligand : protein ratio	L-Tryptophan resonance	Without subtraction	With subtraction	Simulated value
1 : 0	2, 5, 6	6.09 ± 0.02		
	4	6.07 ± 0.03		
	7	6.06 ± 0.04		
	Average	6.07 ± 0.02		
3 : 1	2, 5, 6	ND	4.5 ± 0.6	
	4	2.2 ± 0.2	4.6 ± 0.3	
	7	2.19 ± 0.01	5.23 ± 0.02	
	Average	2.2 ± 0.2	4.8 ± 0.4	5.01
5 : 1	2, 5, 6	ND	5.11 ± 0.3	
	4	3.01 ± 0.05	5.25 ± 0.01	
	7	3.08 ± 0.09	5.58 ± 0.09	
	Average	3.05 ± 0.04	5.3 ± 0.2	5.29
7 : 1	2, 5, 6	ND	5.17 ± 0.3	
	4	3.66 ± 0.01	5.29 ± 0.08	
	7	3.50 ± 0.07	5.1 ± 0.1	
	Average	3.58 ± 0.08	5.2 ± 0.1	5.46
10 : 1	2, 5, 6	ND	5.95 ± 0.06	
	4	4.51 ± 0.01	5.74 ± 0.02	
	7	4.35 ± 0.01	5.74 ± 0.03	
	Average	4.43 ± 0.08	5.8 ± 0.1	5.62

Note. ND: Diffusion coefficients could not be measured for this resonance due to severe overlap with the protein.

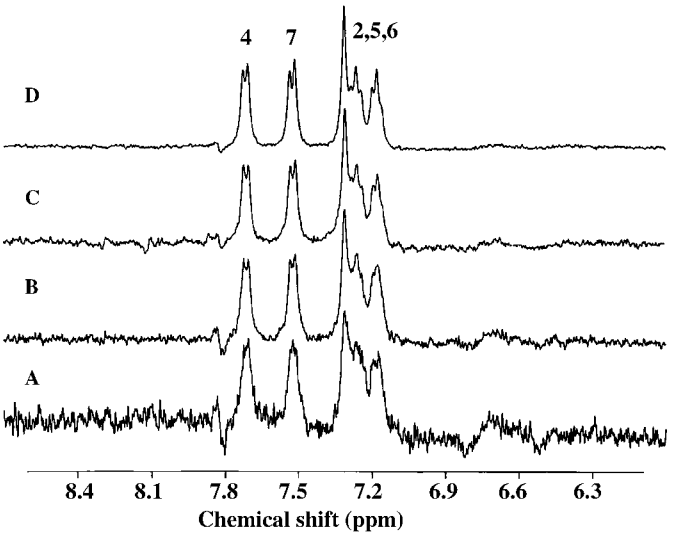


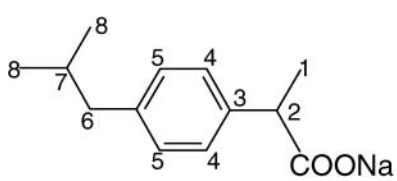
FIG. 3. Aromatic region of L-tryptophan : HSA mixtures after spectral subtraction of the HSA protein in molar ratios of: 3 : 1 (A), 5 : 1 (B), 7 : 1 (C), 10 : 1 (D).

combined with diffusion edited NMR (*31*). Using a similar approach, Tillett *et al*. used ^{15}N-filtered diffusion experiments to measure protein/ligand interactions (*32*). However, these approaches are limited in their applicability, as they require the use of a specially labeled ligand.

An alternate way to eliminate the contributions to the resonance integrals arising from the protein background is by subtraction of the free protein spectrum from those of the protein/ligand complex. Therefore at each molar ratio studied, BPPLED spectra measured for a solution of the free protein were subtracted from the corresponding spectra of the L-tryptophan : HSA solutions. Figure 3 shows the stacked plot of the aromatic region of the NMR spectra of L-tryptophan : HSA solutions after subtraction of the protein background. The effectiveness of this strategy for minimizing the contributions from the protein background can be discerned by comparison with the spectra shown in Fig. 2 and from the diffusion coefficients in Table 1 calculated from the same spectra following background subtraction. The resonances from the tryptophan 2, 5, and 6 protons, which were obscured by the HSA resonances, are clearly resolvable in the spectra shown in Fig. 3. The L-tryptophan diffusion coefficients calculated by integration of the subtracted spectra in Table 1 are in better agreement with the values predicted by our simulation. Background subtraction also gives rise to poorer precision for the ligand diffusion coefficients derived from the subtracted spectra. The higher errors are expected due to the decrease in signal-to-noise ratio resulting from spectral subtraction. However, the precision is also affected by subtraction artifacts that arise from the electronics of our older spectrometer, and better results should be anticipated using a newer instrument with more robust and stable electronics.

Diffusion measurements of ibuprofen binding to HSA. The diffusional behavior of ibuprofen (Scheme 2) upon binding to HSA has been characterized recently in the literature (*15, 33*). As part of this analysis of affinity NMR, it was constructive to reproduce some of the experiments reported in the previous study using spectral subtraction to determine whether protein background is truly negligible even at these high ligand concentrations.

Ibuprofen has one high affinity binding site on the HSA protein. However, many lower affinity sites have also been reported (*25, 26*). Despite this fact, we used Eq. [3] to predict the ligand diffusion coefficient expected for 1 : 1 binding as a guide to interpretation of the experimental results. The experiments were begun at an ibuprofen : HSA molar ratio of 28 : 1. Below this molar ratio, ibuprofen resonances were not observable in the spectrum even after spectral subtraction. Unlike L-tryptophan, all ibuprofen resonances are directly overlapped with those of the protein, therefore ibuprofen concentrations must exceed those of the protein to be observable and sufficiently resolved for integration. As shown in Fig. 4, for a molar ratio of 28 : 1, only



SCHEME 2

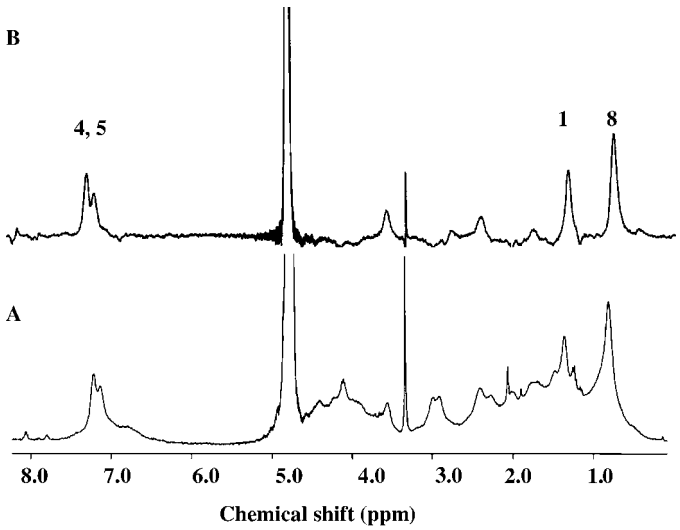


FIG. 4. Spectra of ibuprofen:HSA mixtures at a molar ratio of 28 : 1 without spectral subtraction (A) and with spectral subtraction (B).

the ibuprofen aromatic protons can be observed above the protein background. Without background subtraction, the integrals of the ligand resonances will undoubtedly contain a significant contribution from the protein. Following subtraction of the protein spectrum, the methyl resonances become observable and resolved. When the ibuprofen, to HSA molar ratio is increased to 59 : 1, the remainder of the ibuprofen resonances become apparent in the spectrum and may be integrated without the aid of spectral subtraction. As the molar ratio was increased to a maximum of 140 : 1, all ibuprofen resonance intensities are well above those of the protein and it becomes the dominant species in the spectra (Fig. 5).

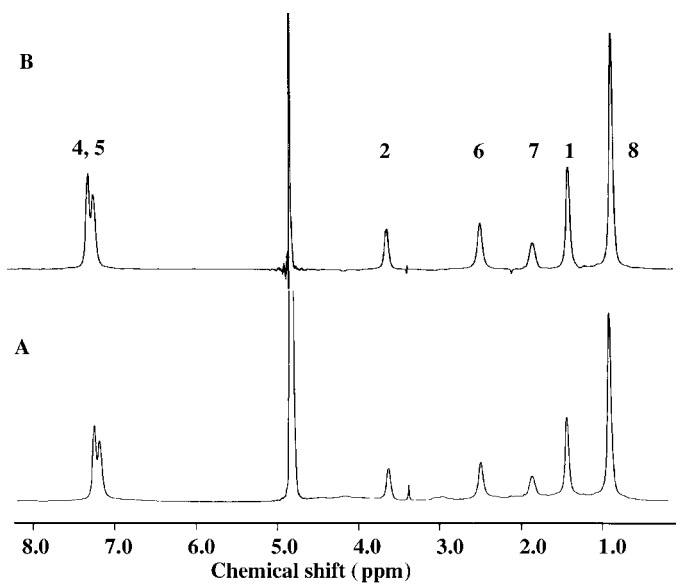


FIG. 5. Spectra of ibuprofen : HSA mixtures at a molar ratio of 140 : 1 without spectral subtraction (A) and with spectral subtraction (B).

Diffusion coefficients obtained without the aid of spectral subtraction were very similar to those previously reported (*33*). Even at a molar ratio of 140 : 1, there is still evidence of protein contribution to the integrated intensity of ibuprofen resonances although it is not visually obvious from the spectrum shown in Fig. 5a. However, even at this large ligand : protein ratio, the protein still contributes to the integrated intensity of the ligand resonances. This can be seen from the linearity of the plots of the natural log of integrated resonance intensity versus the gradient amplitude squared for the 140 : 1 L-tryptophan : HSA solution shown in Fig. 6. The obvious curvature in the data shown in Fig. 6a, obtained without background subtraction, reflects the contribution from the slower diffusing protein. However, the data shown in Fig. 6b by integration of the same spectra following subtraction of the protein background produces a nearly perfect linear fit reflecting a single component, that of ibuprofen.

The diffusion coefficients calculated for ibuprofen with and without spectral subtraction are given in Table 2. The dramatic increase in the diffusion coefficient after subtraction confirms that protein background cannot and should not be ignored even at high relative ligand concentrations. However, as can be seen in Fig. 4b, subtraction of the protein background can produce baseline problems due to subtraction artifacts especially at low ligand : protein molar ratios, which combined with the poorer signal-to-noise ratios of the subtracted spectra lead to higher errors in the diffusion coefficients determined from the spectra with subtraction. As a result, diffusion coefficients for the aromatic and methyl regions produced the most reliable results because these were less affected by the baseline problems.

If one assumes a 1 : 1 binding equilibrium between ibuprofen and HSA, the observed diffusion coefficients predicted by our simulation for the concentration ratios studied will be very close to the diffusion coefficient of free ibuprofen (5.47×10^{-10} m^2/s) at all molar ratios considered because of the large excess of free ligand present in the solution. As evident in Table 2, the diffusion coefficients obtained after spectral subtraction do not approach this value reflecting binding of ibuprofen at additional lower affinity sites. However, the extent of binding would be grossly overestimated if one does not take into account contribution of the protein background to the integrated intensity (and hence the calculated diffusion coefficient) of the resonances of interest.

Dissociation constants were calculated using the ibuprofen diffusion coefficients measured from the subtracted spectra assuming a single binding site with 1 : 1 stoichiometry, an obvious oversimplification for this system. However, the dissociation constants given in Table 2 are consistent with ibuprofen binding at the lower affinity sites on HSA and are in good agreement with values found in the literature for these sites (*27, 28*). The K_d values presented in Table 2 increase regularly with the ligand concentration indicating successive saturation of the available HSA low-affinity binding sites.

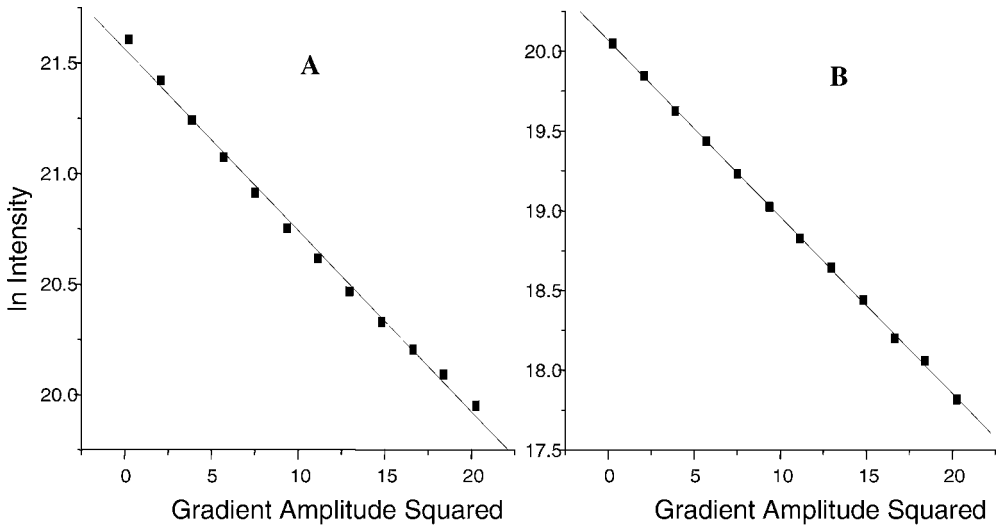


FIG. 6. Plots of the natural log of integrated resonance intensity versus gradient amplitude squared (G^2/cm^2) for the aromatic protons of ibuprofen at a molar ratio of 140:1. The plots constructed without (A) and with (B) of spectral subtraction are shown.

Saturation Transfer

Many protein/ligand binding experiments are performed in water where some form of solvent suppression is needed in order to observe the resonances of interest. For these experiments the conventional method of solvent suppression is presaturation, in which the solvent resonance is irradiated with moderately high power for a short duration. During this process not only is

TABLE 2
Effect of HSA Binding on the Ibuprofen Diffusion Coefficients ($\times 10^{-10}$ m^2/s)

Ligand : protein ratio	Ibuprofen resonance	Without subtraction	With subtraction	Simulated value for 1 : 1 specific binding	Kd ($\times 10^{-3}$ M) assuming 1 : 1 specific binding
1 : 0	4, 5 (Aromatic)	5.47 ± 0.02			
	1 (Methyl)	5.47 ± 0.02			
	8 (Methyl)	5.47 ± 0.01			
	Average	5.47 ± 0.01			
28 : 1	4, 5 (Aromatic)	1.39 ± 0.02	3.15 ± 0.01		
	1 (Methyl)	ND	3.4 ± 0.2		
	8 (Methyl)	ND	3.5 ± 0.2		
	Average	1.39 ± 0.02	3.3 ± 0.2	5.31	1.4 ± 0.1
59 : 1	4, 5 (Aromatic)	2.44 ± 0.02	3.87 ± 0.01		
	1 (Methyl)	1.93 ± 0.01	4.37 ± 0.07		
	8 (Methyl)	2.28 ± 0.01	4.13 ± 0.05		
	Average	2.2 ± 0.3	4.1 ± 0.3	5.39	4.0 ± 0.2
101 : 1	4, 5 (Aromatic)	3.25 ± 0.02	4.32 ± 0.01		
	1 (Methyl)	2.73 ± 0.01	4.77 ± 0.07		
	8 (Methyl)	3.14 ± 0.02	4.50 ± 0.06		
	Average	3.0 ± 0.3	4.5 ± 0.2	5.42	7.7 ± 0.1
140 : 1	4, 5 (Aromatic)	4.1 ± 0.1	4.57 ± 0.02		
	1 (Methyl)	3.90 ± 0.05	4.75 ± 0.01		
	8 (Methyl)	4.22 ± 0.06	4.68 ± 0.03		
	Average	4.1 ± 0.2	4.67 ± 0.09	5.44	11.2 ± 0.4
140 : 1 with saturation	4, 5 (Aromatic)	4.31 ± 0.04	4.43 ± 0.01		
	1 (Methyl)	3.90 ± 0.05	4.60 ± 0.03		
	8 (Methyl)	4.22 ± 0.06	4.62 ± 0.05		
	Average	4.1 ± 0.2	4.6 ± 0.1	5.44	11.2 ± 0.4

Note. ND: Diffusion coefficients could not be measured for this resonance due to severe overlap with the protein.

the solvent resonance irradiated, but protein resonances in the same chemical shift region are also saturated. For large highly coupled molecules such as proteins, saturation transfer to other resonances occurs via spin diffusion along the protein backbone, suppressing the protein signals as well as the solvent.

We tested the effectiveness of saturation transfer for reducing the protein background for the highest molar ratio of ibuprofen : HSA studied (140 : 1). The results, presented in Table 2, suggest that saturation transfer provides some benefit; however, this mechanism of eliminating the protein resonances is not as effective as background subtraction in our hands. At lower molar ratios of ibuprofen to HSA, saturation was counterproductive due to transfer of saturation to the bound ligand, a major pitfall of this method for reducing protein background.

CONCLUSIONS

In this paper we have shown that several factors must be considered when using affinity NMR methods for the analysis of protein–ligand interactions. First, there must be a significant difference in the observed diffusion coefficients of binding and nonbinding ligands. If this criterion is satisfied, optimum concentration ratios of the ligand and protein, as determined by simulation, should be used. Subtraction of the spectrum of the free protein can be used to eliminate the background produced by the protein resonances and yield ligand diffusion coefficients that reflect the true extent of binding. However, subtraction experiments decrease the spectral signal-to-noise and increase the experimental time. When the experiments involving the interaction of ibuprofen with HSA were performed using solvent suppression, saturation transfer to the protein was not sufficiently efficient to eliminate protein background.

EXPERIMENTAL

Chemicals. Ibuprofen (sodium salt), L-tryptophan, and HSA (essentially fatty acid and globulin free) were purchased from Sigma Chemical Co. (St. Louis, MO). All materials were used without further purification. Deuterated water (D_2O, 99.9% D) was purchased from Aldrich Chemical Co. (Milwaukee, WI). All samples were prepared in 0.2 M phosphate buffer at pD 7.47. The buffer was lyophilized twice from D_2O in order to exchange the buffer protons with deuterium. All pD measurements were made with a Fisher Scientific Accumet 10 pH meter (Fair Lawn, NJ) coupled with a Mettler Toledo combination pH microelectrode (Wilmington, MA) calibrated with aqueous pH buffers. The reported pD values are corrected for the deuterium isotope effect using the relationship $pD = pH^* + 0.40$, where pH^* is the pH meter reading (*34*).

Individual diffusion coefficients of L-tryptophan, ibuprofen, and HSA were measured using stock solutions with concentrations of 15, 40, and 0.10 mM, respectively. The solutions used in each titration experiment were prepared by dilution of these stock solutions. In all experiments, the concentration of HSA was kept constant at 0.1 mM. The L-tryptophan titration experiments were performed by increasing the concentration of L-tryptophan from 0.3 mM to 1.0 mM. During the course of the ibuprofen titration experiments the ligand concentration ranged from 2.9 mM to 14.0 mM.

NMR Diffusion Coefficient Measurements

PFG-NMR measurements for all solutions were performed with a Bruker AM 360 MHz NMR instrument using the bipolar pulse longitudinal eddy current delay (BPPLED) pulse sequence (*35*). The instrument was equipped with Nalorac and Bruker 5-mm z-gradient probes with gradient coil constants of 19.0 and 5.34 G/cm · A, respectively. For PFG-NMR measurements of HSA alone, the Nalorac probe was used to generate higher gradient amplitudes. Diffusion measurements of protein/ligand mixtures were made using the Bruker probe because of its greater sensitivity. One-dimensional ^{1}H BPPLED spectra for the L-tryptophan : HSA titration experiments were acquired with 16,384 data points and a spectral width of 6024 Hz, while those for the ibuprofen : HSA experiments were acquired with 8192 data points. To achieve adequate signal-to-noise ratios, a relaxation delay of 1.5 to 2.0 seconds was used and 2,160 transients were coadded at each gradient amplitude.

Following acquisition, the FIDs were transferred to a Silicon Graphics Indigo workstation for data processing using FELIX 97.0 (Molecular Simulations, Inc.). The FIDs were zero-filled to 16,384 data points (Ibuprofen : HSA) and 32,768 data points (L-tryptophan : HSA) and apodized by multiplication with an exponential function equivalent to 1.0 Hz line broadening. For the spectra used in the determination of the HSA diffusion coefficient, 10.0-Hz line broadening was used due to the broad nature of the ^{1}H spectrum of HSA. Following Fourier transformation, the spectral baseline was corrected by fitting selected baseline points to a fifth-order polynomial function. All spectra were referenced to HOD (4.78 ppm).

Subtraction of the protein background was accomplished by performing identical BPPLED experiments in which only HSA was present in the NMR tube and then subtracting each HSA spectrum from an identical BPPLED spectrum containing both protein and ligand. The resultant spectra were integrated and used to calculate the diffusion coefficient of the ligands in the absence of the HSA background.

Calculation of Diffusion Coefficients

Diffusion coefficients were extracted from nonlinear least squares (NLSQ) fits of the integrated resonance intensity measured for a series of BPPLED spectra acquired as a function of the gradient amplitude. The resonance intensity of these integrals, I, and the diffusion coefficient, D, are related according to the following equation:

$$I = I_o \exp[-D(\Delta - \delta/3 - \tau/2)g^2\gamma^2\delta^2]. \qquad [4]$$

In Eq. [4], I is the resonance intensity measured with the BPPLED pulse sequence, I_o is the intensity of the resonance in the absence of a gradient pulse, D is the diffusion coefficient, Δ is the diffusion delay time (0.15 or 0.20 s), which defines the diffusional time scale, and γ is the proton gyromagnetic ratio. The parameters δ (1.2 ms) and g are the gradient pulse duration and amplitude, respectively. The gradient amplitude for titration experiments was varied from 2.67 to 24.03 G/cm. For diffusion measurements of the HSA protein alone, the gradient amplitude ranged from 19.0 to 95.0 G/cm. The delay between the positive and negative gradient pulse, τ, was 1.1 ms and should be set as short as possible. An eddy current delay of 15.0 ms was employed at the end of the pulse sequence to avoid spectral artifacts resulting from residual eddy currents.

The diffusion coefficients reported in Tables 1 and 2 for each resonance are the average of two experimental trials. Diffusion coefficients determined without spectral subtraction, were reported only for those ligand resonances that were easily resolved from the protein resonances. However, after spectral subtraction, diffusion coefficients for some ligand resonances that were previously overlapped could be integrated accurately and are therefore reported. The error associated with each measurement was not calculated using the exponential fitting errors, as these were always less than the trial-to-trial variability. Instead the errors were calculated using the absolute value of the difference between the average and one experimental trial. Because most trials were performed in tandem, the errors in some cases were small compared to relative errors that are normally observed when making diffusion measurements, which are typically in the range from 2 to 5% of the diffusion coefficient value.

Viscosity Measurements

According to the Stokes–Einstein equation, viscosity is inversely proportional to the diffusion coefficient (*36*). Therefore, changes in the diffusion coefficient of the ligand due to viscosity changes of the solution resulting from addition of the protein must be corrected using the relationship

$$D_1\eta_1 = D_2\eta_2, \qquad [5]$$

where D_1 is the diffusion coefficient of the free ligand in deuterated phosphate buffer, η_1 is the viscosity of the buffer, D_2 is the corrected diffusion coefficient of the free ligand in the protein solution, and η_2 is the measured viscosity of the protein/ligand solution. Viscosity measurements were made using a calibrated Cannon–Manning semi-micro viscometer purchased from Cannon Instruments Co. (State College, PA). The temperature was controlled at 298 ± 0.05 K using a VWR 1160 scientific temperature regulator. The kinematic viscosity (V) was calculated by recording the total efflux time (t) with a stopwatch using the equation $V = tB$, where B is the viscometer constant. The absolute viscosity (η) in centipoise (cP) was calculated using the relationship $\eta = V\rho$, where ρ is the density of the solution.

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