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Detection of Site-Specific Binding and Co-binding of Ligands to Human Serum Albumin using ^{19}F NMR

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SUMMARY

Binding and co-binding of various ^{19}F-labeled ligands to human serum albumin (HSA) has been studied using ^{19}F NMR. Specifically shifted resonances in slow exchange with the free resonances are detected for many of the ligands. These specifically shifted resonances can be studied to yield accurate estimates of site-specific binding constants and stoichiometries. In addition, the use of two different ^{19}F-labeled ligands can directly reveal competition for a given site or independent binding at different sites. For instance, it is easily shown that both 5-F-L-tryptophan and 5-F-salicylic acid are capable of binding independently to two sites on HSA at the same time, without the need for any curve-fitting or assumptions. These results demonstrate that the concept of "sites" on HSA is not only useful but is necessary. The technique also reveals allosteric interactions between 5-F-L-Trp and warfarin co-bound to HSA. This technique proves to be a powerful methodology for studying ligand and drug binding to HSA that is free from some of the pitfalls associated with more traditional techniques such as equilibrium dialysis.

Binding interactions between the plasma protein HSA and drugs, fatty acids, and endogenous ligands (such as the heme breakdown product bilirubin-IXα) is an area that has elicited intense study (1–3). These binding interactions are of critical interest for understanding the pharmacokinetics and bioavailability of a wide variety of pharmaceuticals, particularly organic anions, due to the ability of HSA to bind such a wide spectrum of ligands.

Traditionally, detection of binding interactions has been via equilibrium dialysis or some similar technique whereby global concentrations of free and bound ligands are determined. The data are then usually cast in the form of a Scatchard plot (4) or log plot (5) in order to determine site-specific binding (4) or stoichiometric (5) constants. Molecular co-binding of ligands to a protein is determined by measuring the free concentrations of both ligands (if possible) and casting these into a form whereby competitive binding of the ligands can be assessed (3). There are many problems with this approach, some of which have been outlined by Klotz and Hunston (5). The main problem with quantitative analyses of these types of experiments is that one is trying to extract multiple parameters from a rather featureless curve of, for instance, r/C_f versus r or r versus C_f (where r represents the molar ratio of bound ligand to protein and C_f is the free ligand concentration). This problem is especially acute in the case of HSA because its capacity to bind large numbers of a given ligand is so high (2, 3). It should be stressed that this deconvolution of multiple parameters from a given curve is more a mathematical problem than one that is unique to Scatchard plots. Indeed, the same problems are encountered with log plots, in that many different sets of stoichiometric constants will serve to reproduce a given curve, thereby making their unique determination impossible using simple measurements of free and bound concentrations. This conclusion is easy to verify using literature data (see Ref. 6) and simple computer fits (not shown).

An alternative to the indeterminate answers one gets from global measures of ligand concentrations is to utilize techniques that permit direct observation of specific sites[1] on the HSA molecule. One such technique is the displacement of specifically bound fluorescent probes by various drugs. This approach was used to characterize two sites on HSA; site I ligands are represented by warfarin, and site II ligands are represented by L-tryptophan and ibuprofen (see Fig. 1 and Refs. 7–9). The general applicability of this technique is weakened by the fact that induced fluorescence changes caused by co-binding can be large, and it is difficult or impossible to assign a unique fluorescence "signature" to specific sites. Another technique of

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[1] Here and henceforth, reference to sites shall be taken to mean ligand binding areas on the HSA molecule that exist in the protein-ligand complex; these may or may not exist as preformed sites. If two ligands bind to the same "site," it means that they display competitive equilibria at the same amino acid sequences.

ABBREVIATION: HSA, human serum albumin.

some utility is the use of specifically bound spin labels as probes of ligand binding to HSA (10). A third technique is the proton relaxation enhancement of bound paramagnetic molecules (11). It has been shown that certain sites on the HSA molecule can be "labeled" via their NMR relaxation enhancement values (12, 13). These latter two techniques are limited to paramagnetic ligands, however, mitigating their usefulness as general methods.

A more general approach is to utilize fluorine-labeled ligands and monitor specific ^{19}F NMR chemical shifts for the protein-bound and free states. This technique was used by Gerig and Klinkenborg (14) to observe the binding of 5-fluoro-L-tryptophan to HSA. L-Tryptophan is known to bind to one high affinity site on HSA with a binding constant of $\approx 10^4$ M^{-1} (15, 16). Although this technique seems to provide a method for obtaining more specific information than is possible with traditional techniques such as equilibrium dialysis, it has not been utilized to probe albumin binding since Gerig and Klinkenborg's brief report.

In this communication, we report on ^{19}F NMR results obtained for the binding of various fluorine-containing drugs with HSA. We demonstrate the power of this technique for observing site-specifically shifted resonances, for directly observing co-binding of ligands to HSA, and for observing allosteric effects. In favorable cases, accurate estimates of site-specific binding constants and stoichiometries can be made. These experiments provide a valuable complement to the picture of the HSA binding sites generated in the recently determined low resolution crystal structure of HSA (17).

Experimental Procedures

Materials. Globulin-free crystalline HSA (crystallized from fraction V) was purchased from Sigma (St. Louis, MO) and was defatted according to the method of Chen (18), with the exception of replacing HCl by H_3PO_4. The final samples were exhaustively dialyzed against 50 mM sodium phosphate buffer (pH 7.2–7.4). Chloride was excluded from all samples because it is known to bind to HSA with a binding constant of $\approx 10^3$ M^{-1}, thus complicating analysis of the data. HSA concentrations were determined spectrophotometrically, assuming an extinction coefficient of 36,500 M^{-1} cm^{-1} at 279 nm.

The following drugs were purchased from Aldrich (Milwaukee, WI): 5-fluoro-DL-tryptophan, 6-fluoro-DL-tryptophan, 5-fluoro-salicylic acid, and flufenamic acid. Warfarin, ibuprofen, and bilirubin-IXα were purchased from Sigma. Flurbiprofen was a gift from Upjohn Co., and sulindac and sulindac sulfide were gifts of Merck, Sharpe, & Dohme.

All drugs were lyophilized, weighed, and taken up in either ethanol or alkaline solutions as follows. Fluoro-tryptophan, sulindac, sulindac sulfide, and bilirubin were taken up in alkaline solutions. Bilirubin solutions were prepared in the dark. Small aliquots (1–50 μl) of concentrated ligand solutions were added to 1–2 ml of buffered HSA solutions (50 mM sodium phosphate, pH 7.2–7.4), which contained 10% D_2O. In all cases, the maximal change in pH of the final solution was 0.1 unit. Palmitate, flurbiprofen, ibuprofen, 5-fluoro-salicylic acid, and warfarin were taken up in ethanol (in some cases the ethanol was also made basic). For these solutions the ethanol was added to the NMR tubes and rolled to form a thin film, which was then evaporated with a stream of nitrogen. The dried film was then swirled in the HSA solution until dissolution was attained.

NMR measurements. All ^{19}F NMR measurements were made at 338.3 MHz on a home-built NMR spectrometer at the Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology, using a 10-mm ^{19}F probe. The spectra were run without proton decoupling. The reason for this was that the resonances were too broad in the presence of HSA (both free and bound) to observe any coupling effects; moreover, the correlation times of the bound ligands would be in the range where $^{19}F\{^1H\}$ nuclear Overhauser effects would be negative, thus decreasing the observed bound resonance intensity. Usually between 2000 and 8000 transients were recorded for each spectrum, with an interpulse delay of 1.5–2.0 sec and 4096 data points. Spectra were processed with an exponential multiplication corresponding to a line-broadening of between 5 and 20 Hz before Fourier transformation. All spectra were run at a temperature of 20–25°. All resonances are referenced to the position of the free resonance peaks, so that the only chemical shift parameter is the difference between the free and bound resonances. This is sufficient for the purposes of this study, because no conclusions are drawn from absolute chemical shifts. Purity of the ^{19}F-labeled compounds was ascertained by running ^{19}F spectra in the absence of HSA. In all cases, no impurities greater than approximately 1% of the total resonance intensity were detected.

Results

Site-Specific Chemical Shifts

5-Fluoro-tryptophan. The ^{19}F nucleus provides a convenient label for NMR study because it possesses a spin quantum number of ½ and a gyromagnetic ratio 94% that of ^1H, making its detection almost as easy as that of protons. In addition, many ligands are relatively easy to label or already possess a ^{19}F nucleus (such as sulindac or flurbiprofen), such that finding ^{19}F probes for all the various sites on the HSA molecule is quite possible. Because ^{19}F has such a large chemical shift range, in some cases up to 800 ppm (19), it is plausible to assume that a change in the chemical/magnetic environment upon going from the free to the protein-bound state may induce a relatively large chemical shift. This assumption is correct to a degree, as shown below, although the induced chemical shifts (free − bound) observed here range only from about 1 to 8 ppm.

Previously, Gerig and Klinkenborg (14) showed that the chemical shift difference between the free and bound resonances of 5-F-L-Trp is approximately 4 ppm. At this level of resolution, quantitation of the area of the bound resonance is expected to be straightforward and suitable ligand displacement studies should be easy to perform. Spectra representing the interaction of 5-F-Trp with HSA at two molar ratios are shown in Fig. 2. These spectra differ in two respects from the results of Gerig and Klinkenborg (14). In the first case, we did not separate the D- and L-isomers of 5-F-Trp. Because the D-isomer is known not to bind appreciably to HSA (15, 16), it remains a convenient marker along with the free 5-F-L-Trp resonance. The second difference to be noted is that the bound/free peak area ratio is much greater in our spectra, compared with Fig. 1 of Ref. 14. The reason for this is that a large amount of chloride ion was present in the HSA samples of Ref. 14, thus causing some displacement of the bound resonance (15, 16). Our results showed that addition of NaCl to a 5-F-Trp/HSA sample resulted in a considerable decrease of the bound ^{19}F resonance intensity relative to the free.

Interestingly, the chemical shift for the bound resonance in the case of 6-F-Trp is only 1.2 ppm from the free peak (not shown). The reason for the large difference between the two positional isomers is difficult to ascertain. It is unlikely to be due solely to ring current shift differences, because these shifts in proteins are usually ≤1.5 ppm (20). Because the shift for the 5-F-Trp is so much larger, we utilized it exclusively in these studies.

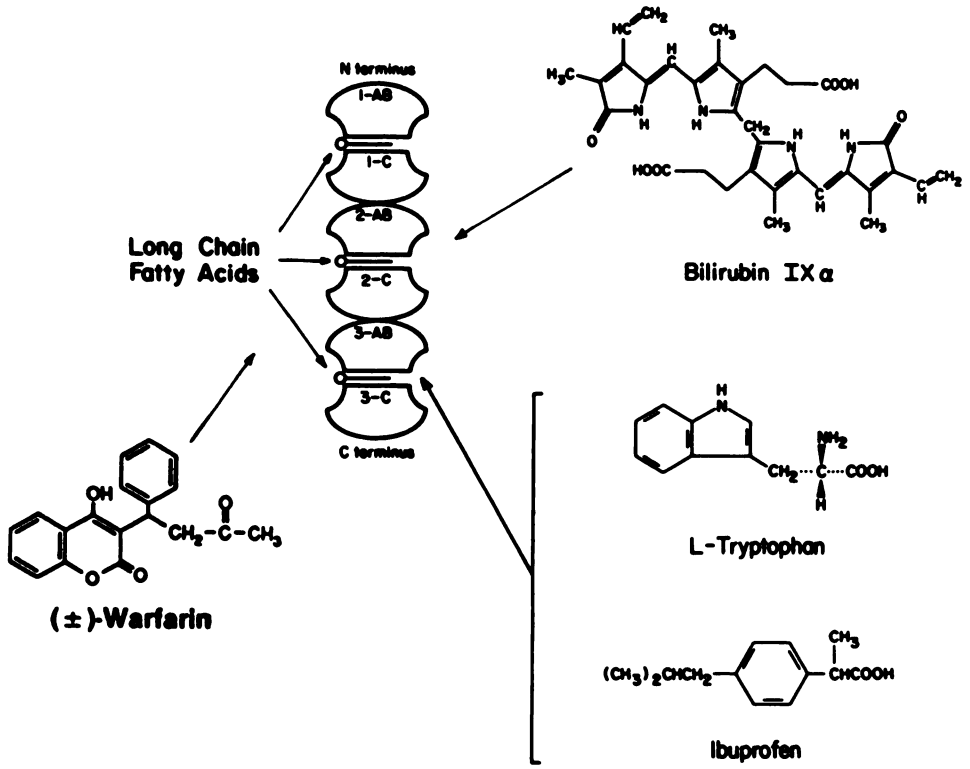

Fig. 1. Schematic diagram of HSA showing the approximate loci of the purported sites for fatty acids, bilirubin, L-tryptophan, and warfarin (adapted from Fig. 9, Ref. 1, and Fig. 8, Ref. 2).

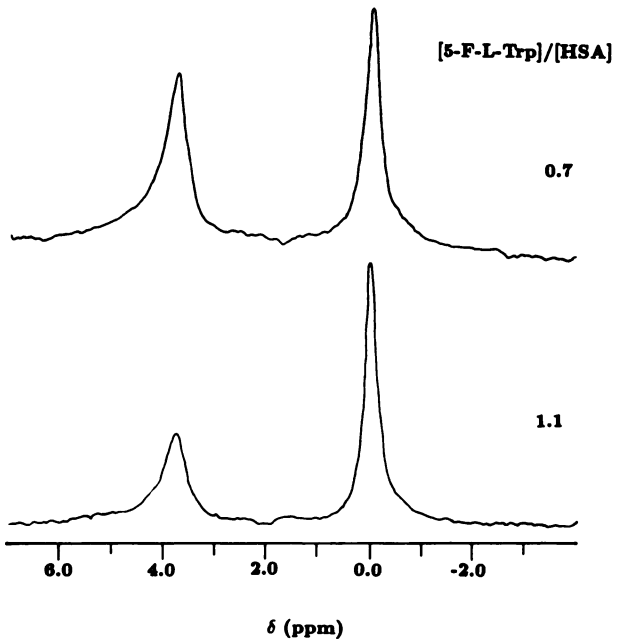

Fig. 2. ^{19}F NMR spectra for interaction of 5-F-DL-Trp and HSA (0.7 mM) in buffer. The peak at 0.0 ppm is due to free 5-F-L-Trp and 5-F-D-Trp.

The stoichiometry of the site represented by the bound resonance in Fig. 2 can be probed simply by increasing the molar ratio of 5-F-Trp to HSA. If increases in the concentration of an ^{19}F-labeled ligand cause further increases in the bound resonance intensity relative to the HSA concentration, then it can be said that that particular resonance represents more than one "site" (i.e., if the bound concentration exceeds the HSA concentration). Conversely, if increases in the ligand concentration never result in a bound ligand/HSA ratio of >1, then the bound resonance represents one specific site. In the case of the two F-substituted-Trp ligands (5-F- and 6-F-), the ratio of bound F-L-Trp/HSA never exceeds approximately 0.9, indicating that the shifted peak represents one site. In addition, the free resonance signal tends to get narrower as the ratio of 5-F-Trp/HSA is increased, implying a higher concentration of unbound ligand.

Binding constants for the site represented by the bound resonance can be estimated in the following fashion. If one assumes that the free peak represents nonspecifically bound ligand plus free ligand, then an equilibrium constant can be calculated for the specific site by assuming that the equilibrium constant for the specifically bound site is much greater than the equilibrium constant for the nonspecific sites. If both areas are easy to quantitate, the goodness of this assumption can be tested. Thus, the following expression can be used in favorable cases where both resonance peaks are readily integrable:

$$K_{eq} = \frac{\frac{A_b}{A_t} \times [\text{Ligand}]_{total}}{\frac{A_f}{A_t} \times [\text{Ligand}]_{total} \times ([\text{HSA}]_{total} - \text{numerator})}$$

where A_b, A_f, and A_t represent the bound, free, and total resonance areas. Utilization of the above equation for over 10 different samples of 5-F-Trp plus HSA yielded K_{eq} values for the specific site of $1.8–4 \times 10^4$ M^{-1}. These values compare quite favorably with the numbers determined by equilibrium dialysis for L-trp (15). (Here the concentration of 5-F-L-Trp is assumed to be ½ that of the total concentration of 5-F-DL-Trp.) It should be pointed out that this approach is not dependent on any of the assumptions implicit in more traditional measurements, namely, independent site binding and multiparametric analysis of featureless curves with relatively few data points. On the other hand, it is dependent on the assumption of relatively weak nonspecific binding. If this assumption is in-

correct, then the measured K_{eq} is only a lower limit. A very accurate bracketing of K_{eq} is possible by utilizing the intercept of a conventional Scatchard plot, because this represents the upper limit for the site-specific K_{eq}. More quantitative considerations can be made using NMR-determined rate constants; however, these will not be presented here.

Other fluorinated ligands. L-Tryptophan is a representative ligand for one, relatively well characterized, binding site on the albumin molecule (1–3, 7–9). However, it would be of great interest to develop other fluorine-labeled molecules as probes of the other putative sites on HSA (Fig. 1). Towards this goal, results are presented for four other ligands in Figs. 3–5, 5-F-salicylic acid, flurbiprofen, sulindac, and sulindac sulfide.

Flurbiprofen is an antiinflammatory drug that appears to bind to the same site as tryptophan and ibuprofen (8, 9, 21). Like 5-F-L-Trp, a bound resonance is detected downfield from the free resonance (Fig. 3). However, the induced shift is only 1.3 ppm downfield from the free resonance. The ratio of the bound peak/HSA concentration does not appear to increase beyond 1:1, indicating that the bound resonance originates from one specific high affinity site. The two peaks tend to merge and broaden as the ratio of flurbiprofen/HSA increases, indicating that there is a considerable proportion of nonspecifically bound flurbiprofen. Interestingly, as the ratio of flurbiprofen/HSA increases beyond 4, an additional peak appears approximately 3 ppm upfield of the free peak. This indicates that there exists another site(s) on HSA for flurbiprofen, in slow exchange with the free peak, which must have a relatively low affinity constant(s). In the case of flurbiprofen, the determination of the affinity constant for the primary site is problematic because at ratios of flurbiprofen/HSA ≤1 essentially 100% of the ligand is bound. This indicates that the binding constant must be at least $\approx 10^6$ M^{-1}.

Salicylate is thought to bind to a site other than the tryptophan site (3, 22). Shown in Fig. 4 are spectra representing the interaction of 5-F-salicylate with HSA. As the ratio of 5-F-salicylate/HSA increases, additional resonance intensity develops about 1.3 ppm downfield of the bound peak. The downfield signal represents free 5-F-salicylate as well as a substantial amount of nonspecifically bound material, as indicated by the broadening of the signal. This broadening is not due to a relatively rapid exchange between the free and bound signals of 5-F-salicylate, because it would be present at lower 5-F-salicylate/HSA ratios. The broad component only starts to appear after the 5-F-salicylate/HSA ratio increases above 1. Again, as is the case with flurbiprofen, essentially 100% of the 5-F-salicylate is bound at ratios near 1, indicating that the binding constant of the primary site must be on the order of or greater than 10^6 M^{-1}. At higher 5-F-salicylate/HSA ratios (equal to 4 and 5; not shown), the downfield resonance sharpens and completely predominates, indicating that the bound component represents a single site.

Sulindac is another antiinflammatory drug that possesses an intrinsic fluorine label. Shown in Fig. 5a are spectra representing the interaction between sulindac and HSA. At low sulindac/HSA ratios, there is one very broad peak, indicating intermediate exchange rates and/or relatively small induced shifts between the free and bound components. As the ratio of sulindac to HSA increases to greater than 1, additional resonance intensity develops about 4.5 ppm downfield of the main resonances. At a ratio of sulindac/HSA of 6, additional resonance intensity appears at about 2 ppm downfield from the main signal. The main signal intensity also starts to sharpen, implying the ratio of free/bound ligand is increasing. Interpretation of these data is complicated by the fact that sulindac appears

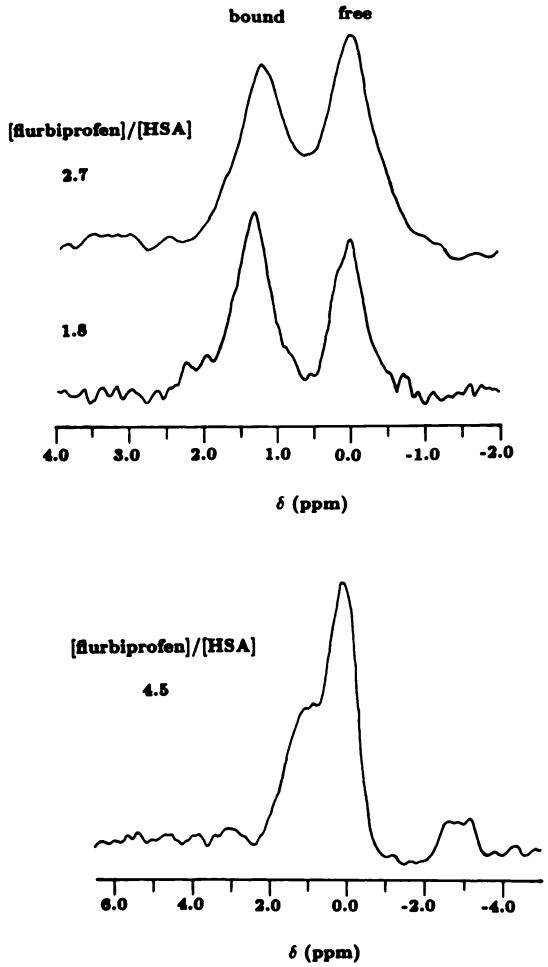

Fig. 3. ^{19}F NMR spectra representing the interaction of flurbiprofen and HSA (0.9 mM) in buffer. At a flurbiprofen/HSA ratio of 1, only the bound peak is seen.

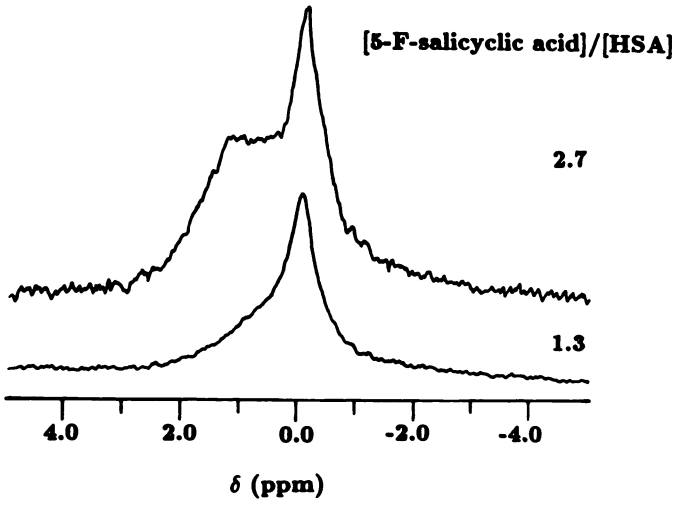

Fig. 4. ^{19}F NMR spectra representing the interaction of 5-F-salicylate acid and HSA (0.7 mM) in buffer.

to bind with positive cooperativity to HSA (23). In any case, it indicates that there are multiple binding sites on HSA for sulindac, some of which are in slow exchange with the free resonances. This feature is exhibited even more powerfully in spectra representing the interaction of sulindac sulfide and HSA.

Shown in Fig. 5b are spectra for HSA and sulindac sulfide, the active metabolite of sulindac (23). It can be seen that there are five peaks resolved at a sulindac/HSA ratio of 1. This indicates that there are at least five different slow exchange chemical shift environments. Sulindac sulfide displays a higher binding affinity for HSA than does sulindac (23), which may explain why there are more resolved peaks in this case as compared with sulindac (Fig. 5a). Each of these has the potential of serving as a marker for different binding regions on HSA. Preliminary data with displacing ligands reveal that these peaks can be selectively displaced.

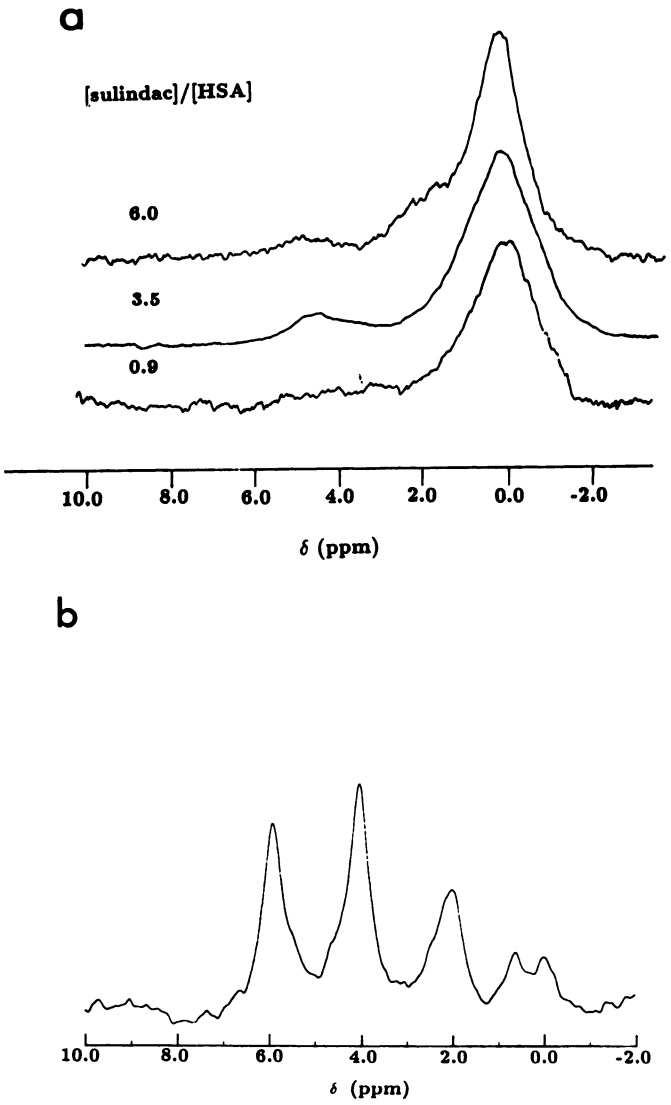

Fig. 5. a, ^{19}F NMR spectra representing the interaction of sulindac and HSA (0.9 mM) in buffer. b, ^{19}F NMR spectrum representing the interaction of sulindac sulfide and HSA (0.9 mM) in buffer. The ^{19}F NMR spectrum of free sulindac sulfide under conditions of proton decoupling was one sharp ($\Delta\nu_{1/2} \approx 2$ Hz) peak with no detectable impurities. Thus, the five resolved peaks are not attributable to impurities.

Additional data representing the interaction between flufenamic acid, which presumably binds to the L-Trp site (7, 8), and HSA are not shown due to the fact that no specifically shifted resonance was observed. The ^{19}F resonance was asymmetrically broadened to a large degree, however (≈220 Hz at a ratio of 1.4:1 with HSA), indicating that there is an induced shift that is too small to resolve. This may relate to the fact that flufenamic acid possesses a CF_3 group rather than having the fluorine directly on the aromatic ring as is the case with the other ligands. Addition of ibuprofen to this solution results in considerable sharpening and symmetrization of this resonance, indicating displacement of flufenamic acid.

Co-Binding of Ligands to HSA

One intriguing potential of the ^{19}F NMR technique is the possibility of directly observing the co-binding of different ligands to HSA. This would enable one to directly determine competition for similar sites without the pitfalls associated with the deconvolution of multiple parameters from measurement of global free and bound concentrations. That this goal is quite feasible is demonstrated in Fig. 6 for co-binding of 5-F-Trp and 5-F-salicylate to HSA. It is seen that at a ratio of 5-F-salicylate/5-F-L-Trp of 1.3 essentially all of both ligands are bound, although there is a slight decrease in the signal intensity of 5-F-Trp. As more 5-F-salicylate is added to the NMR tube, the displacement of 5-F-Trp increases. This indicates either that there is a negative coupling of binding constants for the equilibria involving 5-F-salicylate, 5-F-Trp, and HSA (24) or that the secondary site of 5-F-salicylate is the same as the primary site of 5-F-L-Trp, or both.

The displacement experiments need not be performed with two ^{19}F-labeled ligands. Shown in Fig. 7 are experiments detailing the direct competition of 5-F-L-Trp and ibuprofen for a binding site on HSA. It is seen that when the ratio of ibuprofen/5-F-L-Trp is 2, essentially all the specifically bound resonance intensity is gone. This contrasts with the competition between

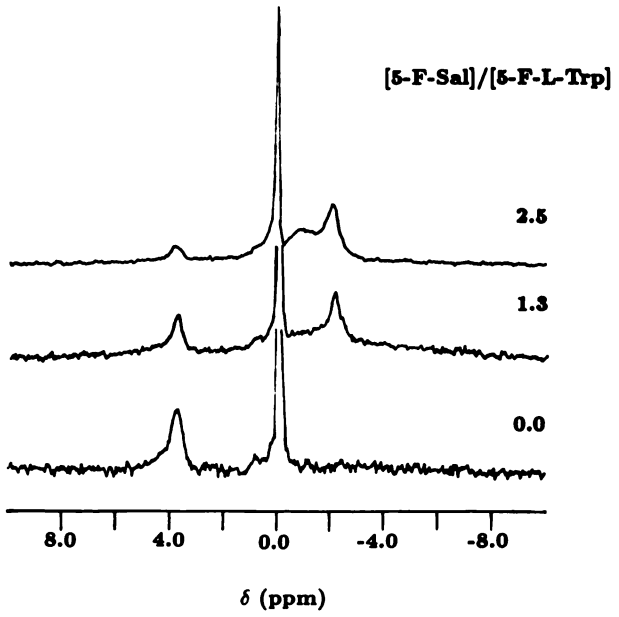

Fig. 6. ^{19}F NMR spectra representing the interaction of 5-F-Trp, 5-F-salicylate, and HSA in buffer. Both the HSA and the 5-F-L-Trp concentrations were constant at 0.45 mM. Number of transients increased from 4500 at the *bottom* to 7432 at the *top*.

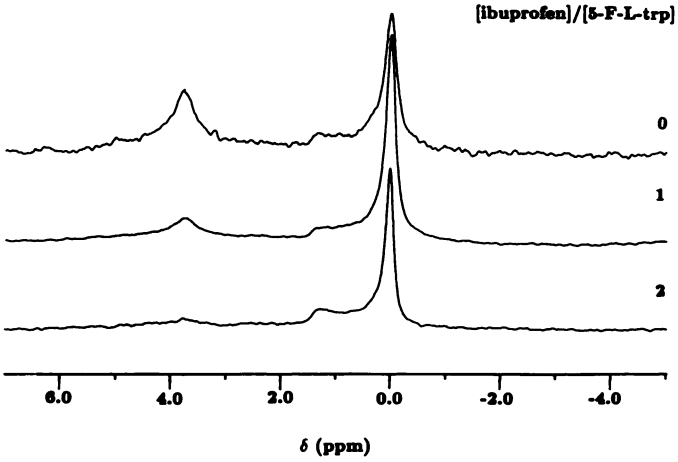

Fig. 7. ^{19}F NMR spectra representing the interaction of 5-F-Trp, ibuprofen, and HSA in buffer. Concentrations of HSA and 5-F-L-Trp were constant at 0.7 and 0.73 mM, respectively.

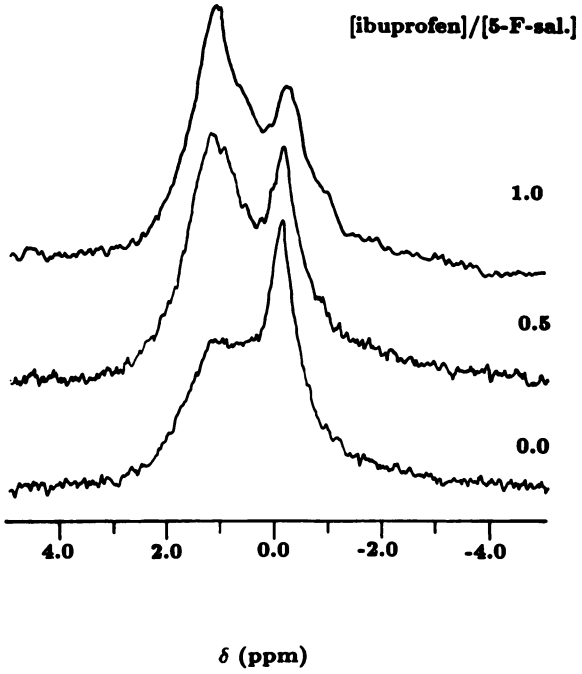

Fig. 8. ^{19}F NMR spectra representing the interaction of 5-F-salicylate, ibuprofen, and HSA in buffer. The HSA and 5-F-salicylate concentrations were constant at 0.7 and 1.8 mM, respectively.

5-F-salicylate and 5-F-L-Trp, where even at a ligand ratio of 2.5 there is still a considerable proportion of the specifically bound 5-F-Trp resonance. Interestingly, as more ibuprofen is added to the solution, an additional peak develops about 1.2 ppm downfield from the free peak. This is quite intriguing and suggests that binding of ibuprofen either creates an additional site for 5-F-L-Trp or opens up a binding site for 5-F-D-Trp. This point needs additional data to be clarified.

The companion experiments detailing competition between 5-F-salicylate and ibuprofen for binding sites on HSA are shown in Fig. 8. It is seen that ibuprofen decreases the intensity of the bound component of 5-F-salicylate and increases the free component. Again, this can represent either competition of the secondary site of ibuprofen for the primary site of 5-F-salicylate or negative cooperativity (as noted above for 5-F-Trp plus 5-F-salicylate). Increasing the ratio of ibuprofen/5-F-salicylate to 4 (not shown) causes a large increase in the free component, but bound intensity is still detectable.

Many other ligand displacement experiments were undertaken with 5-F-Trp and HSA. These ligands included bilirubin, warfarin, palmitate, and the diamagnetic gallium(III) analogue of a hydrophobic iron(III) chelate, which is a potential magnetic resonance imaging contrast agent (12, 13). These results are compiled in Fig. 9. Of all the ligands studied it is obvious that ibuprofen is the only one whose primary site is also the L-tryptophan site. All the others exhibit negative cooperativity or "free energy coupling" (25) to a degree. However, addition of the fatty acid palmitate to 3.6 equivalents almost completely displaces 5-F-Trp, implying that a tertiary site for palmitate may be the 5-F-Trp site. This effect has been noted earlier (15).

Allosteric Effects

Hydrodynamic and proton exchange experiments indicate that albumin is quite flexible (1–3, 26, 27). This flexibility may quite possibly be related to its ability to bind such a wide spectrum of ligands. Evidence of the conformational pliability of HSA can be demonstrated in the interactions between HSA, warfarin, and 5-F-Trp. It can be seen in Fig. 10 that addition of warfarin to the 5-F-Trp plus HSA solution causes very little displacement of the 5-F-L-Trp bound resonance. The resonance does broaden considerably, even as its intensity remains constant. This implies that a conformational change in the HSA molecule is causing a chemical shift change for the bound 5-F-L-Trp resonance. This is not just an increase in the exchange rate of the bound ligand, because the line width of the free peak does not change. The line widths of the bound resonance increase from 169 to 269 to 342 Hz as the warfarin/5-F-L-Trp

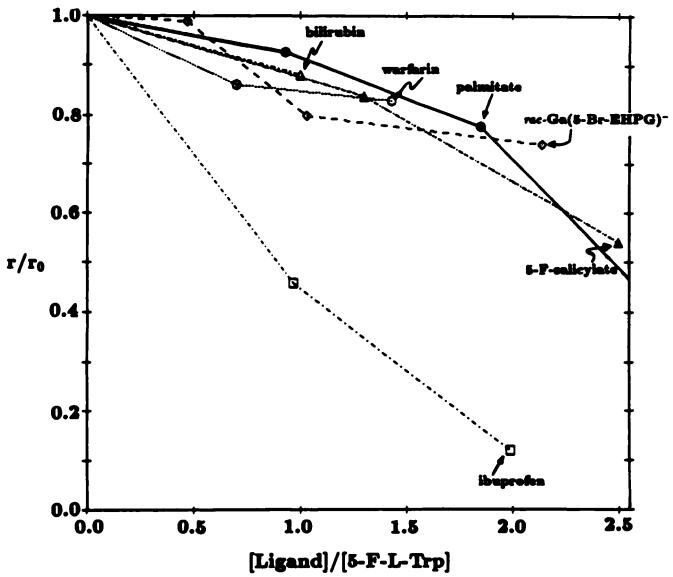

Fig. 9. Plot of r/r_0 for co-binding of the ligands shown with 5-F-L-Trp and HSA (see also Fig. 1 for their purported sites). The quantity r represents the concentration of specifically bound 5-F-L-Trp divided by the HSA concentration in the presence of the competing ligand, and r_0 represents the concentration of specifically bound ligand divided by the HSA concentration in the absence of the competing ligand. These concentrations were determined by integration of the specifically bound resonance at approximately 4 ppm. *rac*-Ga(5-Br-EHPG)$^-$, *rac*-gallium(III) *N,N'*-ethylenebis[(5-bromo-2-hydroxyphenyl)glycinate].

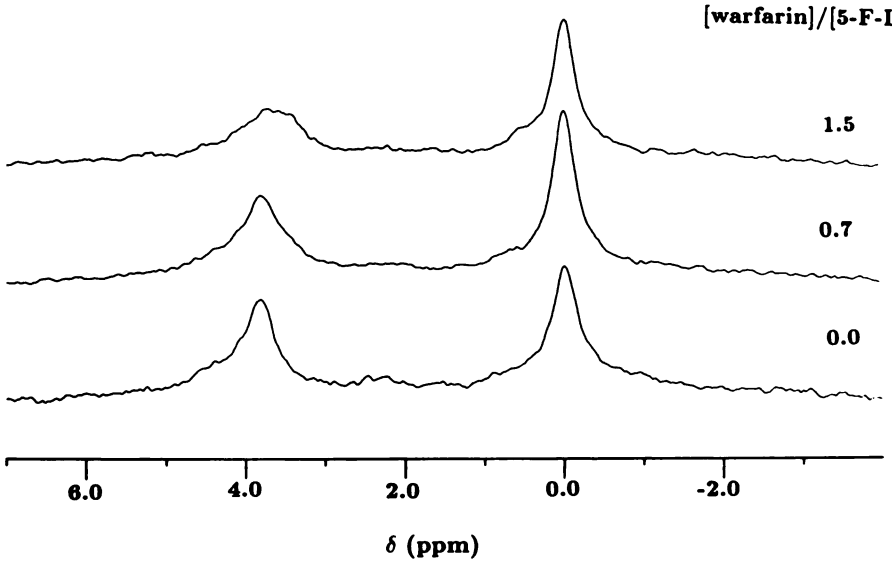

Fig. 10. ^{19}F NMR spectra representing the interaction of 5-F-Trp, warfarin, and HSA in buffer. The concentrations of HSA and 5-F-L-Trp were constant at 0.7 and 0.5 mM, respectively. The peak at approximately 4 ppm is broadened considerably, although its area remains constant.

TABLE 1

Induced ^{19}F NMR chemical shifts for binding of various ligands to sites on HSA

All shifts are reported for data at 338.3 MHz uncorrected for any chemical exchange effects.

Ligand	δ(bound)–δ(free) (ppm)		
	Primary	Secondary	Higher
5-F-L-Trp	3.9		
6-F-L-Trp	1.2		
5-F-Salicylate	−1.3		
Flurbiprofen	1.3		−3.0
Sulindac	<1	4.5	≈2.0
Sulindac sulfide	≈4.0	≈6.0	≈2.0
Flufenamic acid	<1		

ratio increases from 0 to 0.7 to 1.5. (The linewidth of the free resonance remains constant at 135 Hz throughout these titrations.) These changes correspond to a total change of about 0.5 ppm, which is relatively large (see Table 1). It should be noted that in all the other co-binding experiments of 5-F-Trp studied (see Fig. 9) this phenomenon was not seen.

Discussion

The experiments presented here represent the beginning of an attempt to develop an ^{19}F NMR technique that will enable the determination of site binding interactions in HSA by direct observation of specifically shifted resonances. It is anticipated that, as more fluorine-labeled ligands are studied, suitable probes for all the alleged sites will be found. The technique is simple and powerful. For instance, it is obvious from Fig. 6 that there exist two different primary sites on the HSA molecule for 5-F-L-Trp and 5-F-salicylate. This result is easy to observe directly and requires no assumptions of any sort. A further important conclusion that can be drawn from these experiments is that the concept of "sites" on the HSA molecule has validity and indeed is necessary to explain these results. This conclusion is readily apparent in the recently determined crystal structure of HSA, in which salicylic acid is shown localized in two binding regions, one in subdomain IIA (originally identified as IA in ref. 17; personal communication, Dan Carter) and one in subdomain IIIA, which is probably the L-tryptophan site (17). Based upon our results in Fig. 6, it can be seen that the site localized in subdomain IIA must be the primary site for salicylic acid, whereas that in subdomain IIIA is a secondary site. Although some of the competitive experiments performed here have already been reported in the literature (cf. Refs. 1–3, 7–9, 24), the ^{19}F NMR results are less equivocal because they result from direct observation of the effects of co-binding on one specific site.

A further demonstration of the power of this technique is the ability to determine the stoichiometry of the primary sites. This determination can only be ascertained from equilibrium dialysis experiments through extensive curve fitting of r versus C_f. Although we will not present the simulations here, it is easy to show that curve fitting to a high affinity site with a stoichiometry of 2 is very difficult to separate from an assumption of two independent sites. HSA is particularly difficult in this regard because of its ability to weakly bind large numbers of ligands in excess of its primary high affinity sites. This high capacity binding makes it necessary to fit plots of r versus C_f to three (and usually more) parameters. Because the functional forms of these plots are relatively simple, the unique determination of binding constants and stoichiometries is problematic. Our data indicate that examination of these secondary, and higher, sites may be quite difficult with ^{19}F NMR for some ligands, but one can readily observe binding at the specific primary sites and make conclusions without curve fitting.

The ^{19}F NMR technique represents an additional method for studying ligand-induced conformational changes in HSA and is a valuable complement to other biophysical techniques like electron paramagnetic resonance spin labeling (28, 29). Our finding that warfarin appears to alter the chemical shift of specifically bound 5-F-L-Trp is particularly remarkable because the primary sites for the two ligands are thought to be in separate domains of the protein. The tryptophan site [Sudlow site II (7–9)] is known to be in domain IIIA, whereas warfarin is thought to bind to domain IIA (2). Thus, the conformational change appears to extend over a distance of approximately 60 Å or more (see ref. 17).

The primary disadvantage of this technique relates to the insensitivity of NMR in general. At the ^{19}F concentrations

employed here (approximately 1 mM), acquisition of the data for one particular molar ratio requires at least 2-hr of scanning time (and preferably more for doing quantitative integrations) using a standard ^{19}F NMR probe at 300–400 MHz. In our view, the specific molecular information obtained makes the time required a secondary consideration.

Many other experiments based upon these observations can be envisioned. For example, localization of amino acid residues comprising these sites could be determined via selective $^{19}F\{^1H\}$ and $^1H\{^{19}F\}$ nuclear Overhauser effect difference experiments. It has been shown that all 16 histidine residues present in the primary structure of HSA can be observed in the ^1H NMR spectrum of HSA (30). Thus, one could perform selective nuclear Overhauser effect experiments between various His residues and the bound ^{19}F resonance. In addition, one could observe ^1H difference spectra for saturation of the ^{19}F resonances. These and other ^{19}F NMR experiments are currently underway.

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