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Correspondence of Fatty Acid and Drug Binding Sites on Human Serum Albumin: A Two-Dimensional Nuclear Magnetic Resonance Study

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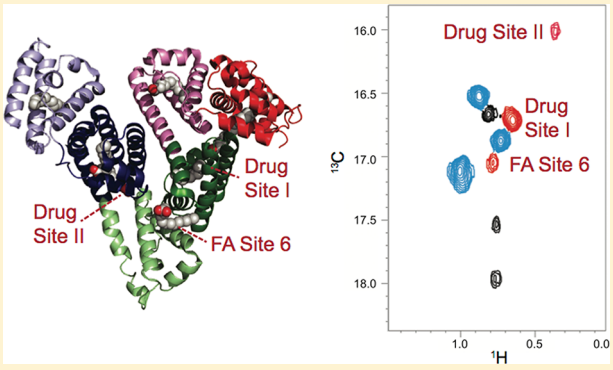
Supporting Information

ABSTRACT: The ability of human serum albumin (HSA) to bind fatty acids (FA) in multiple sites has been revealed by many studies. Here we detect and characterize nine individual binding sites by two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy of 18-[^{13}C]-oleic acid (OA) complexed with HSA. We characterize site-specific FA binding by addition of (i) different FA molar ratios (from 1:1 to 4:1 OA:HSA) to observe the order of filling and occupancy of binding sites; (ii) methyl-β-cyclodextrin, as a FA acceptor, to observe the dissociation of FA; and (iii) drugs (with known binding sites in the crystal structure) to reveal the correspondence of three NMR peaks with sites in the crystal structure. At 1:1 and 2:1 OA:HSA ratios, three sites were shown to fill sequentially. These high-affinity sites were well resolved from additional sites (one medium-affinity and five low-affinity) observed at 3:1 and 4:1 OA:HSA ratios. Methyl-β-cyclodextrin extracted OA from individual sites in the reverse order of filling. FA bound in three low-affinity sites were displaced by drugs shown to bind in crystalline HSA to FA sites 7 and 3 (Sudlow's drug sites I and II, respectively) and FA site 6. With this strategy, 2D NMR spectral analysis permits site-specific characterization of the binding of drugs and FA and provides a sensitive probe of the mutual effects of FA and ligand binding.



Human serum albumin (HSA) is the most abundant protein circulating in plasma, as well as a major component of most extracellular fluids, including interstitial fluid and lymph. In addition to its role in transporting nonesterified fatty acids (FA), its diverse functions include maintenance of colloid osmotic pressure and sequestration of toxins and oxidants.[1] The FA binding sites on HSA also accommodate a broad spectrum of endogenous anionic and neutral compounds such as bilirubin, hemin, bile acids, eicosanoids, cationic metals such as copper(II) and nickel(II), and a variety of hydrophobic electronegative drugs.[1] Prior to the elucidation of the crystal structure of HSA,[2,3] two distinct primary drug binding sites, Sudlow's drug sites I and II, were identified and characterized for their ability to reversibly bind anionic drugs such as warfarin and phenylbutazone.[4,5] Multiple FA hydrophobic binding sites and their asymmetric distribution have been most clearly revealed by crystal structures of Curry et al.[3] X-rays have also revealed sites for other endogenous and several exogenous ligands. Crystal structures with compounds bound in drug sites I and II, such as warfarin, phenylbutazone, diazepam, and propofol,[6] are particularly useful for our current studies because FA are also known to bind to these sites.

The crystal structure of HSA also provides a basis for gaining a detailed description of existing drug binding, for the development of new drugs, and for predicting how altered plasma binding of a drug might affect the volume of distribution, rate of clearance, and plasma half-life of drugs.[7−9] Changes in drug−protein binding occur in a variety of disease states, including renal disease, hepatic cirrhosis, hypoalbuminemia, and epilepsy.[9] In hyperbilirubinemia, bilirubin binding to albumin becomes saturated in neonates. The resulting unsequestered bilirubin may cause kernicterus, an irreversible and debilitating neurological condition.[10−12] Therefore, it is vital also to study combinations of ligands under various physiological conditions, especially with the ubiquitous FA ligand present.

The remarkable design of HSA for binding FA has been revealed by many studies. A continuing goal is to illuminate details of the individual binding sites in the physiologically relevant solution state. FA are typically present at ratios of 0.5−2 mol bound per mole of albumin,[1,13] and up to 6:1 in states of extreme fasting, diabetes, or cardiovascular disease.[1,13−17] With the use of ^{13}C enrichment of specific carbons of the FA molecule (primarily the carboxyl carbon), we have previously characterized the interactions of albumin with the FA carboxyl groups for FA with chain lengths from 8 to 26 carbons.[18−26]

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Oleic acid (OA) is the most abundant FA in the plasma, and the binding properties are very similar to those of palmitic acid.[1,18,19,27,28] The location in binding sites of different FA in the X-ray structure is also similar.[29,30] A more detailed description of binding was obtained for myristic acid (MA) (C14:0) with ^{13}C enrichment at three different carbons. Multiple distinct binding environments of the FA and high molecular mobility were seen at all three carbons.[20] More recently, our work combined unique information obtained from NMR spectroscopy with the HSA crystal structure by identifying binding sites with the use of site-specific HSA mutations and drug competition of bound FA.[27,28]

This study exploits the enhanced resolution of a sensitivity-enhanced $^{1}H-^{13}C$ HSQC pulse sequence[31] by using [^{13}C]-methyl-labeled OA, overcoming the incomplete resolution of FA binding sites in one-dimensional (1D) NMR spectra.[32] We probed the relative affinities of FA in a site-specific manner by three approaches: (i) sequential addition of FA to observe the order of filling and occupancy of binding sites, (ii) addition of cyclodextrin to observe dissociation of FA from HSA, and (iii) addition of drugs that are known to bind to specific low-affinity sites to displace FA molecules and perturb individual chemical shifts.

EXPERIMENTAL PROCEDURES

Materials. Wild-type HSA purified from serum (96% FA-free), diazepam, diflunisal, ibuprofen, phenylbutazone, warfarin, and methyl-β-cyclodextrin were purchased from Sigma Aldrich Co. (St. Louis, MO). Deuterium oxide ($^{2}H_2O$, 99.9%), dimethyl sulfoxide-d_6 (D, 99.9%), 18-[^{13}C]-oleic acid (99%), and 1-[^{13}C]-palmitic acid (99%) were from Cambridge Isotopes (Andover, MA).

[^{13}C]-Labeled FA Stock Solutions. [^{13}C]FA stock solutions (K^+ salt) of both 18-[^{13}C]-oleic acid and 1-[^{13}C]-palmitic acid were prepared as previously described.[27]

HSA Solutions. Wild-type HSA (lyophilized powder) was dissolved in NMR buffer [50 mM KH_2PO_4 and 50 mM NaCl (pH 7.4, adjusted with NaOH)] and stored at 4 °C for up to 1 week. The potassium salt is used for the buffer to complement the use of KOH in the preparation of the [^{13}C]-labeled FA. The concentration of HSA was determined by optical density (OD), where $\varepsilon_{279} = 30810\ M^{-1}\ cm^{-1}$.[33]

HSA–OA and HSA–OA–Drug Complexes. All drugs were dissolved in DMSO-d_6 to make a 100 mM stock and stored at 4 °C for up to 1 week. Complexes were prepared as described previously.[6,18,27] For HSA–OA complexes (final volume of 550 μL), the appropriate quantity of K^+ salt [^{13}C]OA was added to a known volume of hydrated HSA (final concentration of 0.5 mM) with 10% (v/v) D_2O as an internal reference for the NMR lock signal. To study drug interactions, the appropriate quantity of drug was added to the prepared HSA–OA complex to yield a final volume of 550 μL (pH 7.4).

HSA–OA–MβCD Complexes. A 200 mM methyl-β-cyclodextrin (MβCD) stock solution was prepared in ddH_2O. For HSA–OA–MβCD complexes, the appropriate quantity of MβCD was added to the prepared HSA–OA complex to yield a final volume of 550 μL (pH 7.4).

NMR Data Collection and Data Processing. NMR measurements were taken on a Bruker DMX 500 MHz 5 mm inverse triple-resonance probe at 298 K. For 1D experiments, the chemical shifts of the FA peaks were calibrated using the internal reference of the ε-Lys/β-Leu carbon signal at 39.47 ppm.[34] For two-dimensional (2D) experiments, ^{1}H chemical shifts were calibrated by using sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an external reference, and ^{13}C chemical shifts were indirectly calibrated by calculating the spectral reference (SR) value of ^{13}C from the SR value of ^{1}H (^{13}C:^{1}H ratio of 0.251449519).[35]

A standard Bruker pulse sequence, a sensitivity-enhanced $^{1}H-^{13}C$ heteronuclear single-quantum coherence (HSQC) gradient pulse sequence, "invietgpsi", was applied to all complexes with OA.[31] This pulse sequence is a 2D 1H/X correlation via double inept transfer with sensitivity improvement, Echo/Antiecho-TPPI gradient selection, and is ^{13}C-decoupled during acquisition. The C–H coupling is set to 130 Hz. The heteronuclear gradient echo allows for efficient water suppression without the need for water presaturation.[31] The ^{1}H dimension was collected with a spectral width of 10.0 ppm centered at 15.0 ppm using 2048 data points for each of the total 96 scans per experiment, with four scans per increment at 298 K. A relaxation delay of 1.3 s was used. The ^{13}C dimension was collected with a spectral width of 5 ppm centered at 4.7 ppm using 96 data points. A standard ^{13}C "zgdc" decoupled pulse sequence was applied to all complexes with either PA or OA.

NMR data were processed and interpreted with XWin-NMR version 2.6 and XWin-Plot. The intensity of the peak at its apex was determined with XWin-NMR version 2.6 after baseline and intensity scaling factor corrections had been performed.

RESULTS

Nine Distinct FA Binding Sites on HSA Are Revealed in 2D NMR Spectra. Previous studies established the correspondence of binding sites represented by the carboxyl resonances of 1D NMR spectra with sites in the HSA crystal structure (Figure 1A). Via titration of [^{13}C]carboxyl-enriched palmitic acid (PA) to HSA at a 4:1 molar ratio (Figure 1B), NMR peaks for three high-affinity and several low-affinity FA binding sites were assigned to crystallographic FA binding sites.[27,28] However, spectral overlap in this 1D spectrum presented a limitation for assignments, showing four peaks when FA are known to be distributed in several other binding

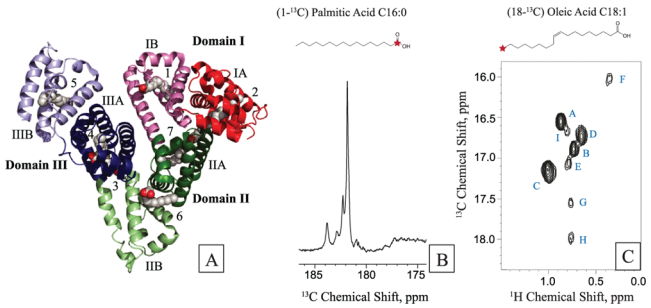

Figure 1. Comparison of the 1D spectra of [^{13}C]palmitic acid and 2D spectra of [^{13}C]oleic acid complexed with HSA. (A) Crystal structure of HSA with seven MA molecules bound, labeled 1–7, domain I (red), domain II (green), domain III (blue), subdomain A (darker shade), and subdomain B (lighter shade) (PDB entry 1E7G). (B) ^{13}C 1D NMR spectrum of 4 molar equiv of [1-^{13}C]PA to 1 molar equiv of HSA, showing only the carbonyl region. (C) $^{1}H-^{13}C$ HSQC spectrum of 4 molar equiv of [18-^{13}C]OA to 1 molar equiv of HSA. The lettering scheme for identifying the peaks is shown. Spectra were recorded at 500 MHz, 25 °C, and pH 7.4 in a 50 mM phosphate, 50 mM NaCl buffer.

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sites.[28] With the use of 2D NMR, our 2D spectra resolved nine individual FA binding sites (Figure 1C). The binding sites are labeled in their order of association with the protein, A–I (see the following section).

Individual binding sites are detected in NMR spectra because the local environments of each binding site are structurally distinct,[20] and the binding site exchange rates are slow on the NMR time scale.[20] The lifetimes of the FA must be >66 ms, a property of FA with chain lengths of >10 carbons. In marked contrast, the more water-soluble medium chain FA (8 and 10 carbons) show fast exchange at ≥20 °C, with a single time-averaged peak.[20] In our 2D spectra of [^{13}C]methyl-enriched OA, the line widths of the two most prominent peaks were 30 ± 5 Hz between 1:1 and 4:1 OA:HSA molar ratios, and the magnitude of the third prominent peak increased from 25 to 35 Hz. This indicates the exchange rate is slow and not affected by mole ratios in this range.

Association of FA with Individual Binding Sites on HSA. With increasing OA:HSA ratios, we employed 2D NMR to establish the relative affinity of OA binding from the filling order, analogous to our previous protocol for 1D NMR studies.[27,28] Starting with a 1:1 molar ratio and 0.5 mM HSA, we found two resonances, peaks A and B (Figure 2A). The greater intensity of peak A indicates that this is the highest-affinity FA site (Figure 2E, plot of absolute intensities). At a 2:1 molar ratio, three peaks were exceptionally well resolved, peaks A–C (Figure 2B). The most intense peak was peak A, and peaks B and C were of similar lower intensities, suggesting slightly lower affinities. Together, these peaks represent the three highest-affinity FA binding sites on HSA. At a 3:1 OA:HSA molar ratio, five additional resonances were evident (peaks D–H). The most prominent peak, D (Figure 2C), has an intensity similar to those of the three highest-affinity sites (Figure 2E). At a 4:1 molar ratio, nine well-resolved peaks are observed (Figure 2D), with the one additional peak designated as I. The last resonances to emerge are identified as low-affinity FA sites, consistent with both their low occupancy and their order of filling. Above a 4:1 molar ratio, no additional sites were detected and the spectra exhibited an overall deterioration of resolution. Counterproductive overlap in the spectral region with the most intense peaks (peaks A–E and I) was observed (Figure S1 of the Supporting Information). This degree of line broadening could be caused by intermediate exchange and/or protein conformation changes. We have previously observed spectral overlap at high mole ratios with the carboxyl-labeled OA and PA.[18,19]

Dissociation of FA from HSA Binding Sites. To further establish relative affinities of HSA binding sites for OA, we studied the site-specific dissociation of FA from HSA upon addition of a FA acceptor. Pilot studies performed in our laboratory with a high concentration of small unilamellar phospholipid vesicles showed minimal transfer of FA from an OA–HSA complex under conditions feasible for monitoring by NMR (data not shown). Because of its higher affinity for FA than for phospholipids,[36] the acceptor methyl-β-cyclodextrin (MβCD) was chosen.

First, we determined that there were no detectable resonances originating from the natural ^{13}C abundance of MβCD (10 mM) in the region of interest (data not shown). As expected, resonances from 0.5:10 and 1:10 OA:MβCD molar ratios (10 mM MβCD) were seen (Figure 3A,B). The primary resonance at 0.87 (^{1}H) and 16.2 ppm (^{13}C) represents the

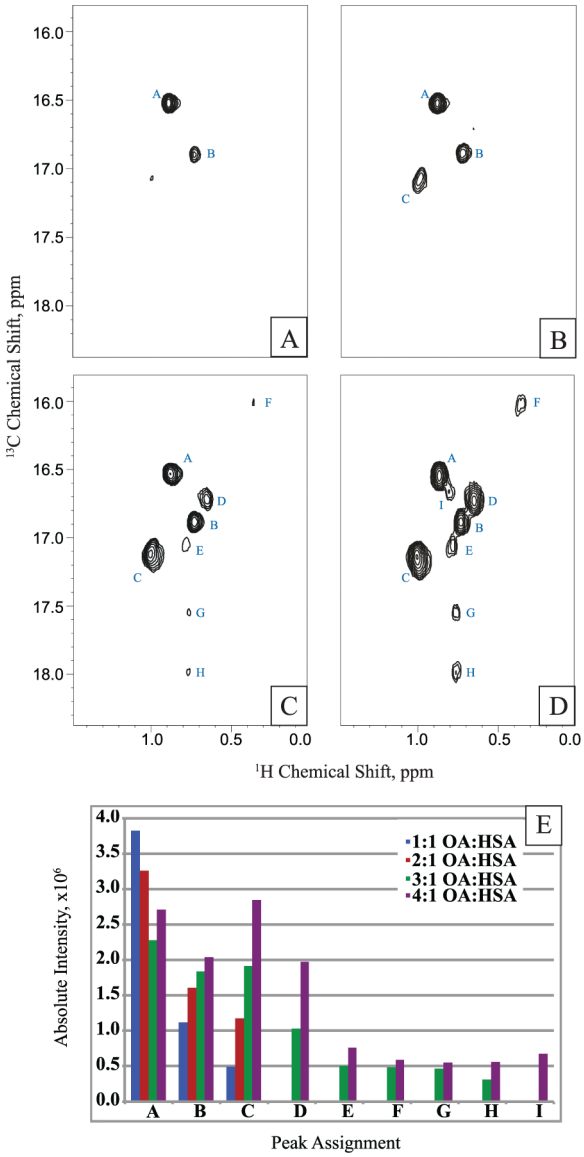

Figure 2. Comparison of increasing molar ratios of OA complexed with HSA. ^{1}H–^{13}C HSQC spectra of HSA with increasing ratios of [18-^{13}C]oleic acid: (A) 1:1, (B) 2:1, (C) 3:1, and (D) 4:1 OA:HSA molar ratios. Spectra were recorded at 500 MHz, 25 °C, and pH 7.4 in a 50 mM phosphate, 50 mM NaCl buffer. (E) Bar graph of the absolute intensity of the individual peaks at varying mole ratios: 1:1 (blue), 2:1 (red), 3:1 (green), and 4:1 (purple) OA:HSA.

majority of the [^{13}C]OA bound to MβCD with minor flanking peaks.

With the addition of MβCD to OA–HSA complexes, our strategy was to look for decreases in the intensity of the OA resonances (site-specific changes). MβCD is typically used at a concentration of 10 mM for the complete extraction of cholesterol from cells.[37,38] In a complex of this final concentration with a 4:1 OA:HSA ratio, there was a complete loss of peak F and decreased intensities of peaks D, E, G, and H (Figure 3C). These data indicated that the FA dissociated from the low-affinity FA site represented by peak F. There was also a decrease in FA occupancy of the sites represented by peaks E, G, and H, which were identified as low-affinity sites from their filling order, and peak D, a medium-affinity site. With an increase to a 50:1 MβCD:HSA ratio, with a final MβCD

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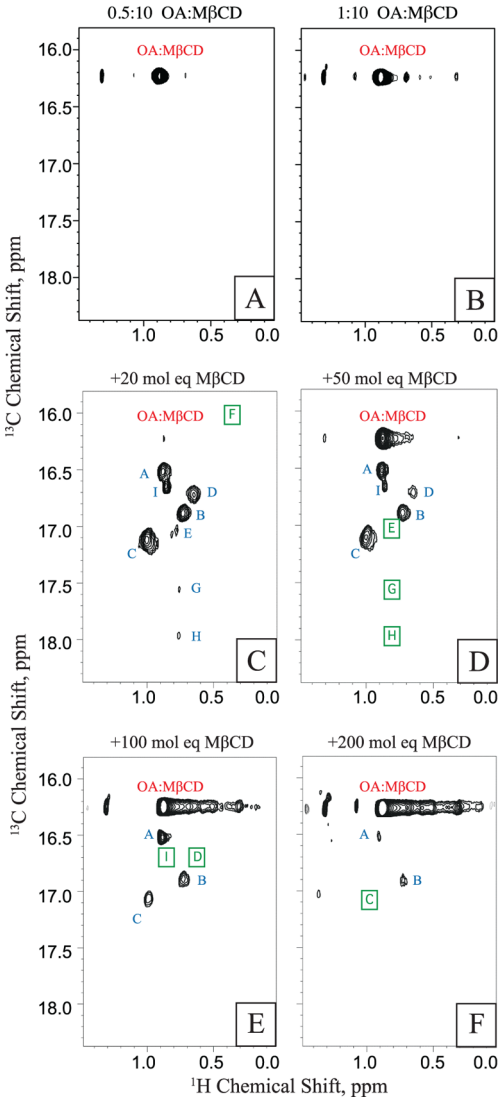

Figure 3. Dissociation of individual FA binding sites from an OA–HSA complex. ^{1}H–^{13}C HSQC spectrum of (A) 0.5 mM OA and 10 mM MβCD and (B) 1.0 mM OA and 10 mM MβCD. ^{1}H–^{13}C HSQC spectra of the OA–HSA complex with increasing molar ratios of MβCD: (C) 20, (D) 50, (E) 100, and (F) 200 molar equiv. The FA peaks labeled in blue indicate association with HSA and in green dissociation from HSA. The OA–MβCD peak is labeled in red. Spectra were recorded at 500 MHz, 25 °C, and pH 7.4 in a 50 mM phosphate, 50 mM NaCl buffer.

concentration of 25 mM (Figure 3D), the spectrum resembled that recorded for the association of a 3:1 OA–HSA complex (Figure 2C). Specifically, we found a complete loss of peaks E, G, and H, a slight decrease in the intensity of peak C, and a marked decrease in the intensity of peaks D and I. The FA bound in these sites either have completely dissociated from HSA or have remained sparsely occupied. The peak representing the OA bound to MβCD has increased intensity, as expected from the increased quantity of MβCD and partitioning of OA from HSA.

At a 100:1 MβCD:HSA ratio, with a final MβCD concentration of 50 mM (Figure 3E), the dissociation spectrum was similar to the association spectrum of the 2:1 OA–HSA complex (Figure 2B). Only peaks A–C representing the three high-affinity FA binding sites were seen. The intensity of each was decreased compared to that seen in the spectrum with 50 molar equiv of MβCD. As expected, there was an increase in the FA occupancy of MβCD. Finally, with the addition of 200 molar equiv of MβCD (100 mM final concentration) to HSA (Figure 3F), only weak intensities were seen for peaks A and B. Therefore, at this very high ratio, FA was removed from high-affinity sites. Note that as the level of MβCD was increased in these experiments, the methyl peak for bound OA became spread over a broad ^{1}H frequency range. This likely reflects altered binding within MβCD molecules that can form larger stacks to solubilize FA. In addition, there could be a heterogeneous mixture of complexes with varying OA:MβCD ratios, as suggested by panels A and B of Figure 3 showing spectra of varying OA:MβCD ratios.

Strategy for Determining the Correspondence of FA Sites and Primary Drug Binding Sites. To establish the correspondence of specific chemical shifts of peaks representing FA binding sites to crystallographic sites on HSA, we employed our previous strategy of site-specific drug displacement.[28] Here, we focused on FA sites that are primary drug binding sites. After experiments with different OA:HSA ratios had been performed, a 4:1 ratio was chosen for systematic studies because it showed nine distinct resonances representing unique FA binding sites on the protein. Although sites with an identical environment in the vicinity of the FA carboxyl could yield an overlapping resonance with the peaks identified, the nine sites likely represent the maximal number of binding sites. With the addition of a non-[^{13}C]-enriched drug or endogenous compound, our strategy was to look for alterations in the intensity of OA peaks. A decrease would indicate partial competition of the drug for the OA site represented by that resonance, while a complete loss of a peak would indicate a near complete displacement of OA by the drug. An increase in the intensity of the peaks could be seen if those sites accommodate the OA displaced by the drug.

Identification of Drug Site I or FA Site 7. To identify Sudlow's drug site I, warfarin, a common anticoagulant, and phenylbutazone, a nonsteroidal anti-inflammatory drug (NSAID) used only for veterinary purposes, were selected. Both have been shown to bind to drug site I in crystal structures, primarily displacing the FA bound at that site, FA site 7 (FA7) (Figure 4A).[6,39] After addition of 1 molar equiv of warfarin (red), the 2D NMR spectrum exhibited a complete loss of intensity of peak D when compared to the spectrum without any drug (black) (Figure 4B). Addition of 1 molar

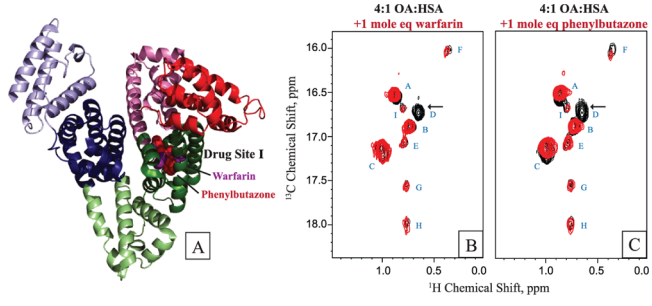

Figure 4. Identification of drug site I. (A) Overlay of binding of warfarin (purple) and phenylbutazone (red) to defatted HSA crystal (PDB entries 2BXD and 2BXP, respectively). ^{1}H–^{13}C HSQC spectra of the 4:1 OA–HSA complex (black) and the 4:1 OA–HSA complex upon addition of (B) 1 molar equiv of warfarin (red) and (C) 1 molar equiv of phenylbutazone (red). Spectra were recorded at 500 MHz, 25 °C, and pH 7.4 in a 50 mM phosphate, 50 mM NaCl buffer.

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equiv of phenylbutazone to an OA–HSA complex resulted in a similar loss of intensity of peak D and small shifts of peaks A and C (Figure 4C). These experiments indicate conclusively that peak D is the resonance associated with the FA bound at drug site I or FA7.

Identification of Drug Site II or FA Site 3. To investigate the correspondence of NMR peaks with drug site II, diazepam, a benzodiazepine used for treating anxiety, and propofol, a general anesthetic, were chosen because both have been crystallized with HSA and bind at drug site II or FA3 (Figure 5A).[6] The addition of 2 molar equiv of diazepam to an OA–HSA complex showed a complete loss of resonance at peak F, suggesting that diazepam binds primarily to the FA site represented by this peak (Figure 5B). Although the intensity of peak F was low, implying a low occupancy by FA, both peaks A and I increased in intensity, which suggests that displaced FA was bound to these sites. Peak D was also altered. While this may indicate a partial displacement of drug site I, the change in peak D may also be attributed to a direct allosteric perturbation of the protein region around this binding site and a shift of intensity of the bound FA.[3,29,40–42] Propofol produced similar changes at even lower mole ratios. Notably, there was a complete loss of peak F after the addition of 0.5 molar equiv (Figure 5C). These results indicate that both diazepam and propofol compete with the same FA for binding at their primary site, which was elucidated from the crystal structures as drug site II. Therefore, this study is able to identify peak F as the resonance associated with drug site II or FA3.

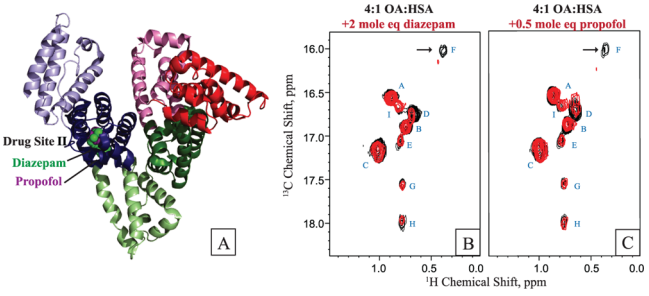

Figure 5. Identification of drug site II. (A) Overlay of binding of diazepam (green) and propofol (purple) in defatted HSA crystal (PDB entries 2BXF and 1E7A, respectively). ^{1}H–^{13}C HSQC spectra of the 4:1 OA–HSA complex (black) and the 4:1 OA–HSA complex (red) upon addition of (B) 2 molar equiv of diazepam and (C) 1 molar equiv of propofol. Spectra were recorded at 500 MHz, 25 °C, and pH 7.4 in a 50 mM phosphate, 50 mM NaCl buffer.

Identification of a Third Drug Binding Site or FA Site 6. To identify FA site 6 (FA6) in our 2D spectra, we first used the NSAID ibuprofen, which binds drug site II and FA6 in the crystal structure (Figure 6A).[6] Addition of 1.0 molar equiv of ibuprofen resulted in the complete loss of low-affinity FA sites, peak F or drug site II, peak G, and peak E (Figure 6B). As an additional probe, we next used diflunisal, another NSAID that binds mainly to drug site II, but also to drug site I and FA6 in the crystal structure of HSA (Figure 6A).[6] With a small amount of diflunisal, the loss of FA bound in drug site II or peak F was evident (Figure 6C). Addition of 2 molar equiv of diflunisal resulted in a decreased FA occupancy of drug site I or peak D and peak E (Figure 6D), while 3 molar equiv caused a complete loss of intensity of these two peaks. Additionally, there was a downfield shift, but no significant loss of intensity of peak G, thus indicating that this peak does not represent the secondary drug binding site FA6. Peaks A, I, and C increased in intensity, suggesting that these sites are binding the displaced FA (Figure 6E). When peaks that have already been defined were excluded, competition experiments with diflunisal saw the complete loss of only peak E. Similarly, experiments with ibuprofen saw the complete loss of peaks E and G. Therefore, it can be concluded that peak E, the common unidentified peak displaced by these two drugs, represents FA6.

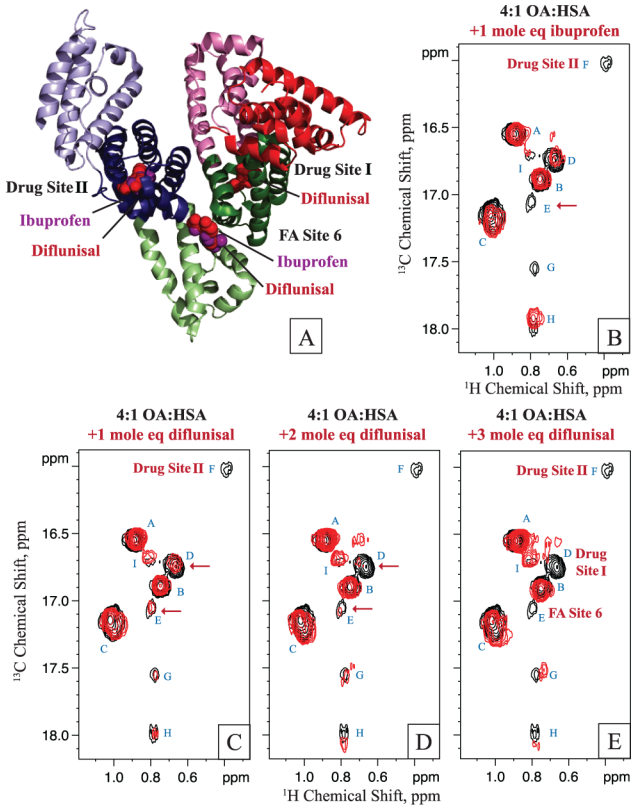

Figure 6. Identification of a third drug binding site. (A) Overlay of binding of ibuprofen (purple) and diflunisal (red) in a defatted HSA crystal (PDB entries 2BXG and 2BXE, respectively). ^{1}H–^{13}C HSQC spectra of the 4:1 OA–HSA complex (black) and the 4:1 OA–HSA complex (red) upon addition of (B) 1 molar equiv of ibuprofen, (C) 1 molar equiv of diflunisal, (D) 2 molar equiv of diflunisal, and (E) 3 molar equiv of diflunisal. Spectra were recorded at 500 MHz, 25 °C, and pH 7.4 in a 50 mM phosphate, 50 mM NaCl buffer.

Rosiglitazone Binding Sites on HSA. Mapping of the primary drug binding sites, drug sites I and II, and FA6, provides a powerful basis for investigating drugs with unknown binding sites on HSA. As an example, we investigated rosiglitazone, a thiazolidinedione widely used for treating patients with type II diabetes. With the addition of 1 molar equiv of rosiglitazone to a 4:1 OA–HSA complex, there was a complete loss of the peak representing drug site II (peak F) and perturbations in the spectral regions of peaks A, D, and I (Figure 7). Compared to the other drugs studied, rosiglitazone had unusually marked effects on other peaks, implying a more heterogeneous environment of FA binding (Figure 7). Our results indicate that rosiglitazone primarily binds to drug site II and its binding has an impact on other sites, including the high-affinity FA site represented by peak A.

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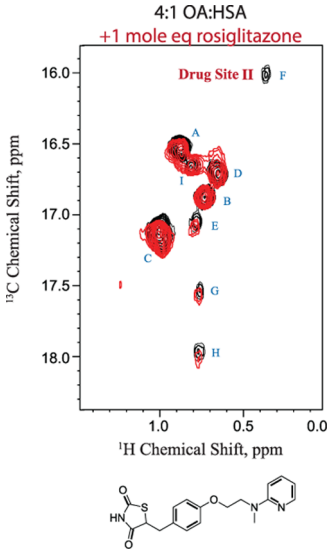

Figure 7. Identification of the rosiglitazone binding site on HSA. ^{1}H−^{13}C HSQC spectra of the 4:1 OA−HSA complex (black) and the 4:1 OA−HSA complex upon addition of 1 molar equiv of rosiglitazone (red). The spectrum was collected at 500 MHz, 25 °C, and pH 7.4 in a 50 mM phosphate, 50 mM NaCl buffer. The structure of rosiglitazone is shown.

DISCUSSION

High Resolution of Individual FA Binding Sites on HSA. After decades of studies of FA−HSA binding with mostly indirect evidence of multiple FA binding sites, the strategies used in this study with 2D NMR spectroscopy have identified nine structurally distinct sites in the solution state and elucidated the order of filling of the sites, which approximates their relative affinities. Individual binding sites are detected in NMR spectra for two reasons. (i) The local environments of the [^{13}C]-enriched carbon in each binding site are structurally distinct,[20] and (ii) the rates of exchange between binding sites are slow on the NMR time scale.[22] The line widths in our spectra were not sufficiently variable to suggest an impact on peak intensity.

The 2D method is powerful for probing FA in a site-specific, individual basis. The observed well-resolved FA resonances (Figure 1) likely correspond to the sites in the crystal structure.[29] However, the solution state with OA has two more sites than were identified in the crystal structure of HSA with long chain FA.[3,6,29] This is not due to binding differences between the FA. Previous reports have shown that FA binding sites are common for both medium and long chain FA.[29,30] Our previous NMR studies estimated the capacity of 7−9 mol of long chain FA per mole of albumin based on the total integrated intensity of FA peaks.[19] Because our NMR studies were conducted within physiological concentrations of both protein (0.5 mM) and ligand (0.5−2.0 mM),[1,13] the additional sites observed in our NMR experiments could originate because of ligand saturating conditions used in crystallographic studies or because of the flexibility of the protein in solution that allows it to adopt different conformations.[43,44]

Site-Specific Association and Dissociation of Individual OA with HSA. This detailed and accurate analysis of the binding of FA to specific sites at differing FA:HSA molar ratios was made possible by the excellent resolution of the methyl peaks of OA in the 2D NMR spectra. In 1D NMR spectra,[18,28] the low resolution and signal-to-noise ratios did not permit definitive assessments of whether there were two or three high-affinity sites. In contrast, these results clearly show that only two sites are significantly occupied with OA at the 1:1 ratio and three sites are occupied with OA at the 2:1 ratio (Figure 2). The 2D NMR spectrum at the 3:1 ratio revealed a new peak (D) with an intensity similar to those of the three highest-affinity sites. Thus, we have identified this peak as a medium-affinity peak. At the 4:1 ratio, peak D had a greater intensity than peak B, signifying a higher occupancy of FA in peak D at this molar ratio. The low intensity and emergence of peaks F−I at the higher molar ratios indicate that these are lower-affinity sites.

The different affinities of FA binding sites, as reflected in their filling order, are especially relevant to the vital physiological role of HSA in the delivery of FA to membranes and tissues. While it can be assumed that FA will dissociate first from the lowest-affinity sites, to the best of our knowledge the site-specific dissociation of FA has not been studied. The acceptor used in these studies, MβCD, is widely used in the pharmaceutical industry as an emulsifier and in research for extracting cholesterol from cell membranes.[45,46] At lower concentrations, MβCD has been shown to extract FA from both model and cell membranes.[36] In our studies, MβCD effectively removed OA from albumin. Manipulation of the MβCD:HSA ratio permitted clear visualization of the progressive loss of OA from lower- to higher-affinity sites, in the general reverse order of filling (Figure 3). This result further supports our conclusion about relative affinities based on the order of filling and illustrates the elegant structural design of albumin that allows control and fine-tuning of the delivery of FA to cells.

Correspondence of Primary Drug Binding Sites and Low-Affinity FA Binding Sites. Several drugs used in this study (warfarin, phenylbutazone, and ibuprofen) bind to HSA at multiple sites.[6] The precise location of drugs bound to HSA and their detailed molecular interactions were not revealed prior to crystallographic studies. Sudlow's drug site I is a large apolar pocket comprised of the six helices of subdomain IIA and a loop−helix structure from subdomain IB.[6,39] Sudlow's drug site II has generally been identified as both FA sites 3 (FA3) and 4 (FA4) in subdomain IIIA of the crystal structure and is a smaller hydrophobic pocket than drug site I.[29] FA4 is a long narrow hydrophobic channel.[6,29] Crystallographic studies of HSA with both FA and drugs bound to drug site II revealed the displacement of FA in FA3, but not FA4.[29]

Crystal structures revealed that a wide range of drugs bind to not only one primary binding site but also secondary binding sites distributed throughout HSA and in the cleft of the protein. In crystallography, these sites are defined on the basis of the electron density of the drug in the binding pocket. The higher the electron density, the more likely that site represents primary binding. Conversely, the lower the electron density, the more likely that site represents a secondary binding site. In the crystal structure, all of these secondary sites overlap with endogenous sites for FA[6,29] and permitted us to design drug−FA competition experiments to make assignments of the major low-affinity FA resonances by 2D NMR. Through FA titration experiments, NMR can show the emergence of the secondary and even tertiary sites with increasing quantities of the drug. Multidimensional NMR provides a high resolution in comparison to the 1D NMR results in which these low-affinity FA resonances were obscured (Figure 1). Here, our competition studies with two known drug site I binding

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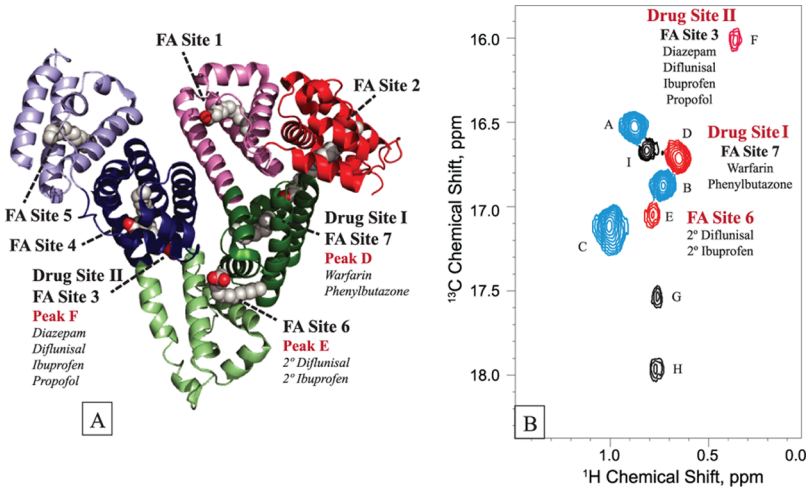

Figure 8. Summary of OA−HSA competition experiments. (A) Crystal of HSA bound to seven labeled MA molecules. Drugs and endogenous compound primary and secondary binding sites are listed (PDB entry 1E7G). (B) $^{1}H-^{13}C$ HSQC spectrum of the 4:1 OA−HSA complex. FA2, -4, and -5 (blue) are primary sites for FA (Figure 2) and are generally not impacted by competition binding. The low-affinity sites, FA3, -6, and -7 (red), were identified through drug competition experiments. The locations of the competition of the drugs and endogenous compounds for their primary and secondary binding are listed. The spectrum was recorded at 500 MHz, 25 °C, and pH 7.4 in a 50 mM phosphate, 50 mM NaCl buffer.

compounds, warfarin and phenylbutazone, identified drug site I or FA7 as peak D (Figures 4 and 8). Drug site II or FA3 was identified as peak F by the competition of diazepam and propofol (Figures 5 and 8).

In addition to sites I and II, the third primary site for drug binding hypothesized from Sudlow's studies with fluorescent probes[4] likely corresponds to FA6. This site is located at the interface between subdomains IIA and IIB at the base of the protein between drug site I (domain IIA) and drug site II (domain IIIA).[29,30] This third site corresponds to a secondary binding site for diflunisal and ibuprofen in the crystal structure.[6] The loss of peak E was seen with the addition of both 3 molar equiv of diflunisal to a 4:1 OA−HSA complex and 1 molar equiv of ibuprofen to a 4:1 OA−HSA complex (Figures 6 and 8). Therefore, peak E represents a third primary site for drugs and corresponds to FA6 on HSA. With the crystal data as a reference, our drug competition experiments have defined the three most important FA binding sites for the study of binding of drugs to HSA: drug site I, drug site II, and FA6.

Significance of the Binding of Drugs to HSA. Knowledge of drug−HSA binding, especially the pharmacokinetic properties that affect the volume of distribution, bioavailability, and elimination half-life of a new drug entity, are important for the development of new molecular entities.[7−9] In this study, a rigorous correspondence of the primary drug binding sites in the crystal structure to NMR resonances (peaks D−F) in the solution state was achieved by drug−FA competition experiments (Figure 8). Our study provides the basis for determining the primary binding site for drugs that compete for binding of FA to HSA.

The assignment of these sites in the 2D NMR spectra of OA−HSA complexes provides a basis for identifying binding site(s) for existing drugs, newly discovered drugs, and lead compounds with unknown binding sites. For example, we studied rosiglitazone, a potent antihyperglycemic agent. Pharmacokinetic studies have shown that rosiglitazone is 99.8% bound to HSA, and because of this, the drug is slowly excreted from the systemic circulation in the time course of more than a week.[9,47] Our novel approach found that rosiglitazone binds to drug site II (Figure 7).

The concentrations of all the drugs in this in vitro study ranged from 0.25 to 2 mM. While the majority of these drugs were used in a concentration range 25−550-fold higher than therapeutic doses,[9] the peak plasma concentrations of diflunisal and ibuprofen in the circulation following therapeutic doses were approximately 0.5 and 0.6 mM, respectively.[9,48] At these pharmacologically relevant concentrations, both of these drugs were competitive with FA at levels that could be detected by 2D NMR. Diflunisal and ibuprofen competition both showed binding at drug site II. The study of FA−HSA binding can be particularly useful for studying the effects and/or binding sites of endogenous HSA binding compounds that have higher circulating concentrations, such as bilirubin.

LIMITATIONS AND FUTURE DIRECTIONS

Our 2D NMR spectroscopic approach is a method for probing site-specific perturbations of binding of FA to solution state HSA at a high resolution. Studies are in progress to determine the correspondence of high-affinity sites in the 2D NMR spectrum to sites in the crystal structure. Because our study was limited to OA, future 2D NMR studies with other [^{13}C]-enriched FA could illuminate interesting differences between FA. The resolution of nine individual FA binding sites and identification of the three most important drug binding sites, drug sites I and II and FA6, provide a strong foundation for further studies of the binding of drugs and endogenous compounds to HSA. This technique and these strategies may also provide novel molecular information about pathological conditions with increased FA levels, such as diabetes.

ASSOCIATED CONTENT

Supporting Information

A figure showing the 5:1, 6:1, and 7:1 OA:HSA molar ratios. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

FA, fatty acid(s); FA3, FA site 3; FA4, FA site 4; FA6, FA site 6; FA7, FA site 7; HSA, human serum albumin; HSQC, heteronuclear single-quantum coherence; MA, myristic acid; MβCD, methyl-β-cyclodextrin; NSAID, nonsteroidal anti-inflammatory drug; OA, oleic acid; PA, palmitic acid; PDB, Protein Data Bank.

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