

Locating high-affinity fatty acid-binding sites on albumin by x-ray crystallography and NMR spectroscopy

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Human serum albumin (HSA) is a versatile transport protein for endogenous compounds and drugs. To evaluate physiologically relevant interactions between ligands for the protein, it is necessary to determine the locations and relative affinities of different ligands for their binding site(s). We present a site-specific investigation of the relative affinities of binding sites on HSA for fatty acids (FA), the primary physiological ligand for the protein. Titration of HSA with [¹³C]carboxyl-labeled FA was used initially to identify three NMR chemical shifts that are associated with high-affinity binding pockets on the protein. To correlate these peaks with FA-binding sites identified from the crystal structures of FA–HSA complexes, HSA mutants were engineered with substitutions of amino acids involved in coordination of the bound FA carboxyl. Titration of [¹³C]palmitate into solutions of HSA mutants for either FA site four (R410A/Y411A) or site five (K525A) within domain III of HSA each revealed loss of a specific NMR peak that was present in spectra of wild-type protein. Because these peaks are among the first three to be observed on titration of HSA with palmitate, sites four and five represent two of the three high-affinity long-chain FA-binding sites on HSA. These assignments were confirmed by titration of [¹³C]palmitate into recombinant domain III of HSA, which contains only sites four and five. These results establish a protocol for direct probing of the relative affinities of FA-binding sites, one that may be extended to examine competition between FA and other ligands for specific binding sites.

albumin mutants | albumin structure | diabetes | fatty acid transport

Albumin is a highly abundant serum protein that serves as a transport vehicle for several endogenous compounds including fatty acids (FA), hemin, bilirubin, and tryptophan, all of which bind with high affinity (1, 2). The protein has long attracted the attention of the pharmaceutical industry because of its ability to bind a wide variety of drug molecules and alter their pharmacokinetic parameters (3). Although most ligands for albumin are hydrophobic anions, heavy metals are also known to bind to the protein (1, 2, 4, 5). This versatility, which arises from the presence of multiple binding sites, means that it is far from trivial to obtain detailed information on ligand binding. It has been especially difficult to assess the interactions between different ligands for the protein even though it is key for our understanding of the role of human serum albumin (HSA) *in vivo*.

HSA is the primary transporter for delivering FA to the tissues and possesses at least seven binding sites for this ligand. Under normal physiological conditions, between 0.1 and 2 mol of FA are bound to albumin, but the molar ratio of FA/HSA can rise to 6:1 or greater in the peripheral vasculature during fasting or extreme exercise (6, 7) or under pathological conditions such as diabetes, liver, and cardiovascular disease (1, 8).

Recent crystallographic analyses of HSA–ligand complexes have revealed the number and locations of binding sites for various ligands including FA (9–12), hemin (13, 14), and thyroxine (15) and

a number of drug molecules (9, 11, 16, 17). However, it has not always been possible to correlate the crystallographic structural information on albumin with binding data gained from a variety of other methods. This correlation is required for a more complete understanding of the ligand-binding properties of HSA, particularly under conditions in which competition between ligands may be significant. The problem of deciphering the affinities of different binding sites is most acute for ligands such as FA, which bind to several different sites on the protein. Because of the necessity of preparing crystals with FA in excess of the total binding capacity of albumin, x-ray structures do not provide direct information about relative affinities.

NMR spectroscopic analysis of the binding of ¹³C-labeled FA to albumin is a sensitive technique for probing albumin–FA interactions and was applied extensively to BSA before the crystallographic analysis of FA–HSA complexes. It has the distinct advantages of using chemically unmodified reagents and allowing the filling of individual binding sites to be followed. Previous studies of BSA (75% identical in amino acid sequence to HSA) have shown that progressive addition of long-chain FA (LCFA) labeled with ¹³C on the carboxyl carbon allowed resolution of several narrow peaks in the NMR spectrum, each of which is linked to a distinct binding environment on the protein (18–20). The number and intensity of these signals depended on the FA/BSA ratio and suggested the presence of two to three high-affinity sites (associated with the earliest appearing peaks) (18, 19).

To assign the highest-affinity sites to regions of the protein, ¹³C NMR binding studies were done with large fragments of BSA that were obtained by proteolysis of the entire molecule. Two high-affinity sites were located in domain III, and a third resided in a fragment that encompassed subdomains IA–IB–IIA (21). However, the methods used did not permit precise location of the FA within the domains. Subsequent studies in several laboratories with recombinant fragments corresponding to the three helical domains of HSA suggest that each domain retains many of the binding properties of the intact protein (22–25), establishing their value for probing ligand binding [although the crystal structure shows that FA sites two, three, and seven span more than one domain and are unlikely to be retained within isolated fragments (11, 12, 15)]. It has generally been found that domain III behaves best in terms of solubility, stability, and ligand binding (22–26).

The precise location of the highest-affinity FA-binding sites on HSA has eluded researchers thus far. We report here an approach

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Abbreviations: FA, fatty acid(s); HSA, human serum albumin; LCFA, long-chain FA.

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that combines structure-based site-directed mutagenesis with NMR analysis of the binding of [^{13}C]palmitate to characterize the relative affinities of the individual binding sites on HSA. Our findings provide the first definitive assignment of two high-affinity FA-binding sites on HSA and establish our integrative experimental approach as a powerful tool for dissecting the complex ligand-binding properties of albumin. The correlation of specific binding sites revealed by crystallography with distinct NMR peaks will also enhance NMR as a tool for solution studies of site-specific perturbations induced by binding of other biomolecules and drugs to albumin.

Materials and Methods

Materials. Recombinant wild-type HSA was the kind gift of Eishun Tsuchida (Waseda University, Tokyo). Delipidated HSA was purchased from Sigma. The potassium salt of [^{13}C]palmitate was purchased from Cambridge Isotope Laboratories (Andover, MA). Deuterium oxide ($^2\text{H}_2\text{O}$; 99.9%) was purchased from Wilmad (Buena, NJ).

Subcloning of Domain III and Mutagenesis of HSA. HSA domain III was subcloned by using PCR into the pPIC9 vector (Invitrogen), which adds an N-terminal signal peptide sequence to direct secretion of the expressed protein into the extracellular medium. N-terminal sequencing of the expressed domain III confirmed that the signal peptide was removed efficiently, resulting in an expressed protein containing residues 382–585. Site-specific mutations were introduced into the full-length HSA ORF cloned into the pHL-D2

expression vector (Invitrogen) (27) by using the QuikChange XL mutagenesis kit (Stratagene). Incorporation of the desired mutation was confirmed by DNA sequencing.

Expression and purification of HSA mutants from *Pichia pastoris* were performed as described (9, 27) with slight modifications. Briefly, after 3–4 days of induction, which results in secretion of the expressed protein into the growth media, nonalbumin proteins were removed from the clarified media by precipitation with 40% (wt/vol) $(\text{NH}_4)_2\text{SO}_4$. HSA then was precipitated with 95% (wt/vol) $(\text{NH}_4)_2\text{SO}_4$. The albumin precipitate was redissolved in deionized water and dialyzed extensively against water for at least 24 h. After defatting with charcoal (28), HSA was purified on a Blue Sepharose 6 Fast Flow column (Amersham Pharmacia Biosciences), followed by gel filtration using Superdex S75 (Amersham Pharmacia Biosciences). Proteins were >95% pure as judged by SDS-PAGE.

A similar protocol was used for purification of recombinant HSA domain III, although in this case defatting was achieved by using a hydrophobic resin (Dextran type IX, Sigma), because the supply of material was limited and this resin is reported to give better yields than charcoal defatting (29). A solution of domain III at 40 mg/ml was incubated with a $20\times$ molar excess of sodium decanoate in 25 mM phosphate (pH 7.0) to displace any hydrophobic ligands derived from yeast. Excess unbound lipid was removed during several cycles of dilution and concentration by using a 5-kDa molecular weight cutoff centrifugal concentrator (Millipore). The pH was reduced to ≈ 3 by addition of HCl and 10 mg of dextran type IX added per mg of protein, followed by slow rotation of the sample for 1.5 h at room temperature. The resin was removed by filtration,

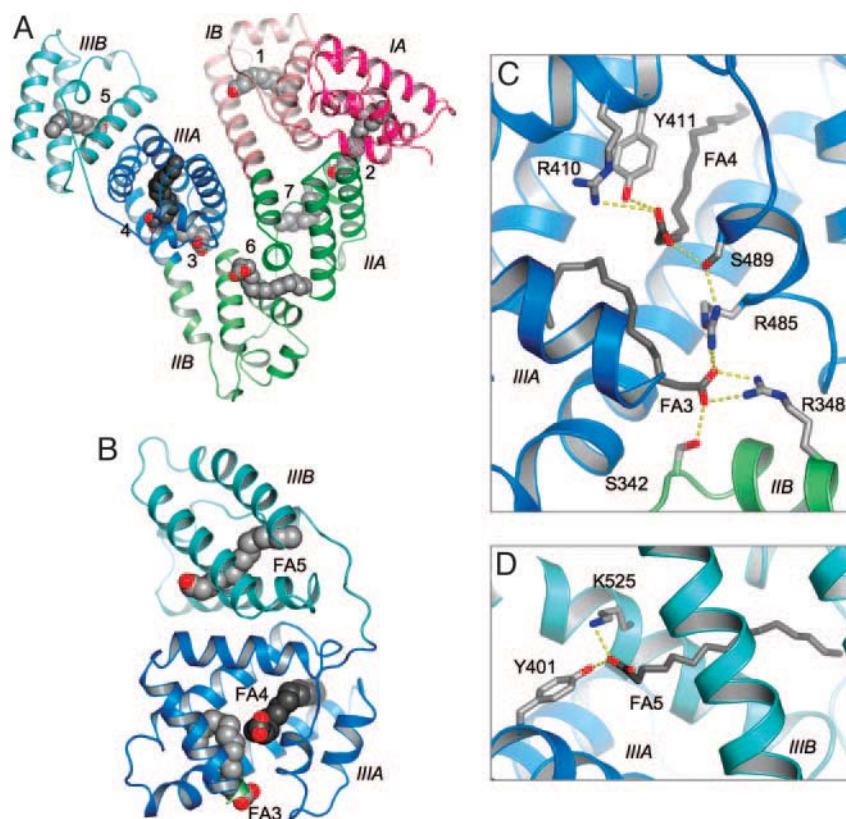


Fig. 1. Structural details of full-length HSA, domain III, and FA sites four and five as revealed by x-ray crystallography. Crystal structure of HSA with palmitate bound (A) and domain III (B) from the same crystal structure, indicating the likely structural details of the recombinant fragment. HSA contains three domains (I, red; II, green; III, blue), each containing A and B subdomains (dark- and light-colored shades, respectively). The crystal structure reveals seven LCFA-binding sites. The FA bound to site four has been darkened to distinguish it from the FA in site three. Shown also is the crystal structure of palmitate bound to FA sites four (C) and five (D). The electrostatic interactions formed between the FA carboxyl and basic residue(s) are indicated by the dashed yellow lines. FA bound to site four forms strong electrostatic interactions with R410 and Y411, whereas K525 forms stable interactions with FA in site five. These figures were made with our palmitate/HSA structure (PDB ID code 1e7h) by using PYMOL (42).

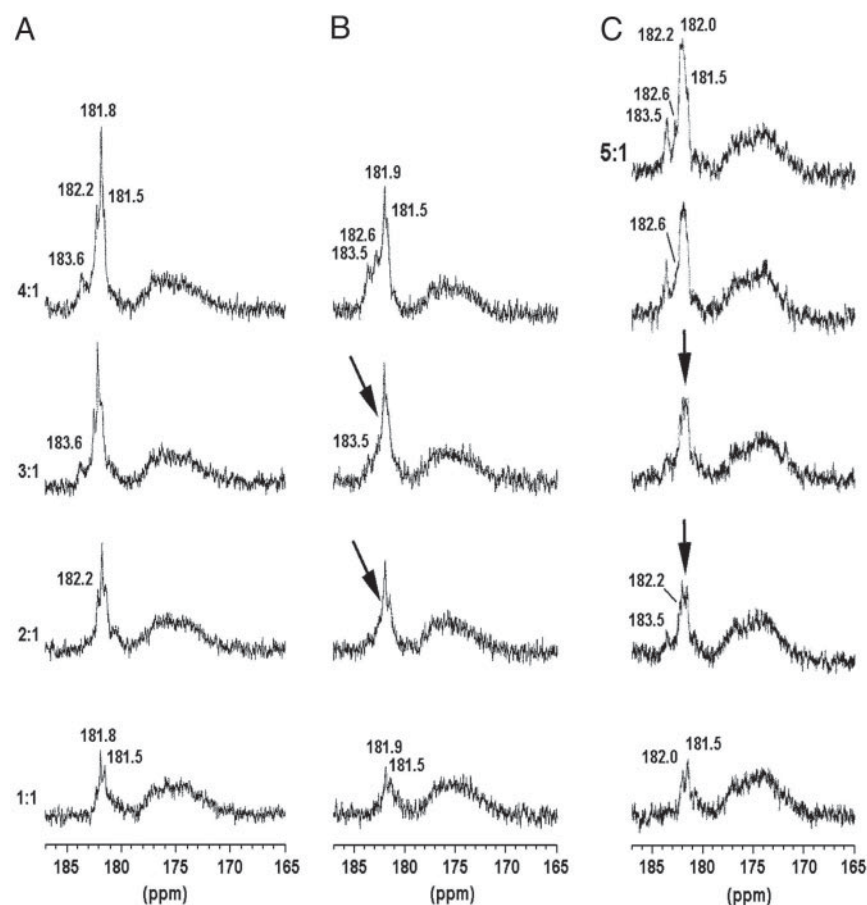


Fig. 2. Titration of wild-type, R410A/Y411A, and K525A HSA mutants with increasing mole ratios of [^{13}C]palmitate. ^{13}C NMR spectra were obtained to study the binding of [^{13}C]palmitate to wild-type HSA (A), R410A/Y411A double mutant (B), and K525A mutant (C). Only the carbonyl region of each spectrum is shown (165–187 ppm). Numbers to the left of each panel indicate the mole ratio of [^{13}C]palmitate to HSA. Values above the labeled peaks indicate the chemical shift in parts per million. For clarity, chemical shifts are labeled as they first appear in the titration. All chemical shifts are labeled at the highest FA/HSA ratio. Missing peaks are marked by arrows. NMR peaks at 181.9 and 182.2 ppm were absent in spectra of the K525A and R410A/Y411A mutants, respectively. The increased intensities of other peaks (182.6 and 183.6 ppm) at 4:1 in each mutant may be caused by displacement of FA to lower-affinity sites.

and the filtrate was restored to pH 7.0 by the addition of NaOH. Tests with wild-type HSA confirmed that this procedure is as effective as the charcoal protocol (data not shown).

HSA Solution Preparation. For studies of FA binding to HSA, solutions of wild-type HSA were prepared essentially as described (18, 19, 30); the protein concentration was determined spectrophotometrically [$\epsilon_{280} = 0.51 \text{ OD}/(\text{mg}/\text{ml})$]. The final concentration of HSA for NMR studies of FA binding was 10–15 mg/ml in a total volume of 2.0 ml. The pH was adjusted to 7.4 with the addition of 0.1 mM KOH. Solutions of HSA mutants and isolated domain III [$\epsilon_{280} = 0.3 \text{ OD}/(\text{mg}/\text{ml})$] were prepared similarly.

Preparation of FA–HSA Complexes. FA–HSA complexes were made according to the protocols that were used to prepare complexes of FA–BSA in earlier studies (18, 19, 30). The stock of [^{13}C]palmitate first was heated to 70°C in hot water until transparent and allowed to cool to 37°C. The desired amount of FA was added to a known volume of HSA, prepared as described above, in a still water bath at 37°C. Sequential mole equivalents of [^{13}C]palmitate were added to the same HSA sample immediately before each NMR measurement, up to 4–5:1 FA/HSA; the sample then was readjusted to pH 7.4 with 0.1 mM KOH and H_2SO_4 and equilibrated to 37°C in a warm water bath before NMR experiments were performed at the same temperature.

NMR Analysis of [^{13}C]Palmitate Binding. All ^{13}C NMR spectra were obtained at 125.31 MHz with a broadband 10-mm probe, 4,000 time domain points, 1.1-sec pulse interval, and broadband ^1H decoupling. $^2\text{H}_2\text{O}$ [10% (vol/vol)] served as the internal NMR “lock” signal. Spectra were processed with a line broadening of 3.5 Hz. The chemical shifts of FA carboxyl peaks were calibrated by using the $\epsilon\text{-Lys}/\beta\text{-Glu}$ peak at 39.47 ppm (30) as an internal standard. FA carboxyl signals appearing in the spectral range of 180–185 ppm were integrated [protein carboxyl groups make a small but constant contribution to this spectral region (18, 30)]; the broad protein carboxyl signal centered at ≈ 175 ppm was set to an intensity of 1.0.

Results

Addition of ^{13}C -labeled FA to recombinant wild-type HSA resulted in several FA carboxyl peaks representing distinct binding environments for FA within the albumin molecule, as observed previously for BSA (18, 31). Preliminary studies of the binding of [^{13}C]LCFA to wild-type HSA that was isolated from plasma showed the same pattern of resonances as the results herein (30). To assign individual ^{13}C NMR peaks to specific binding sites in the crystal structure (Fig. 1A and B), it was crucial to optimize resolution in the FA carboxyl region. We therefore compared the spectra of wild-type HSA with palmitate (C16:0) and oleate (C18:1) (data not shown), which are the two most common FA transported by albumin in plasma (32). Titrations of molar equivalents of [^{13}C]palmitate and [^{13}C]oleate to HSA in solution produced very similar NMR spectra, but the peaks

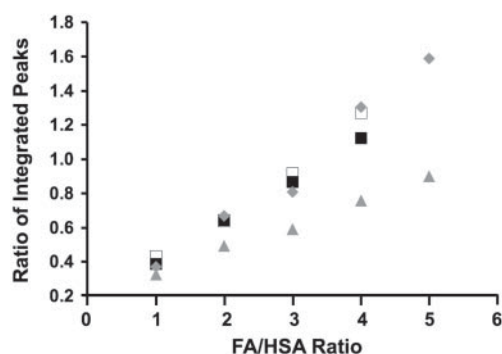


Fig. 3. Total amount of [^{13}C]palmitate bound to wild-type and mutant HSA. The region from 160.0 to 190.0 ppm was integrated for each ^{13}C NMR spectrum of wild-type and mutant HSA. The ratio of integrated FA carbonyl peaks (centered at ≈ 182 ppm) to protein carbonyl signal (centered at ≈ 175 ppm) was calculated and plotted against the FA/HSA ratio. Control experiments were performed for complexes of oleate–HSA (open squares) and palmitate–HSA (filled squares). The total amount of [^{13}C]palmitate bound to R410A/Y411A (filled diamonds) and K525A (filled triangles) were plotted also. The data are generally similar for R410A/Y411A and wild-type HSA bound with either oleate or palmitate. Total FA binding to K525A was reduced significantly.

were better resolved at all mole ratios of palmitate/HSA. Consequently, [^{13}C]palmitate was chosen for all subsequent experiments. At 1:1 palmitate/HSA, two peaks were present (181.5 and 181.8 ppm); a third peak appeared at 182.2 ppm when the ratio was increased to 2:1 (Fig. 2A). At 3:1 and 4:1, a fourth peak was seen at 183.6 ppm. Relative peak intensities reflect the FA occupancy of different sites, and the observed order of appearance of peaks suggests that the peaks at 181.8, 182.2, and 181.5 ppm correspond to the three highest-affinity LCFA-binding sites in HSA. (In the case of partially resolved peaks that cannot be integrated separately with accuracy, peak heights represent approximate intensities).

To assign specific FA carboxyl peaks that were obtained with addition of FA to specific binding sites, we mutated FA-coordinating residues at sites identified from the crystal structures of FA–HSA complexes. We focused on sites within domain III, because the availability of a recombinant fragment corresponding to this domain, which is known to fold autonomously (22, 24, 26), permitted us to follow up observations made with intact HSA in the context of a simpler binding system (see below). We introduced amino acid substitutions of surface residues, which were known to be important for coordination of the carboxyl group of bound FA (11, 18, 31) and were deemed less likely to disrupt protein folding. Two mutant HSA proteins were produced: a double mutant (R410A/Y411A) and a single mutant (K525A) that have lost key FA carboxyl-coordinating residues in FA sites four and five, respectively (Fig. 1C and D) (11, 12).

^{13}C NMR spectra obtained for complexes of [^{13}C]palmitate with wild-type HSA and the two mutants are compared in Fig. 2. The spectrum of wild-type HSA (Fig. 2A) contains one peak (182.2 ppm) that is absent in the spectra of the R410A/Y411A double mutant (Fig. 2B). Loss of this signal in the mutant is most evident at FA/HSA ratios of 2:1 and 3:1 and, interestingly, coincides with increased intensity of the peak at 183.6 ppm and with the appearance of a broad peak at 182.6 ppm. This peak is not observed in wild-type HSA. The most intense peak observed for wild-type HSA (181.8 ppm) is absent or diminished from the spectra of the K525A mutant (Fig. 2C). This mutant also shows an increase in intensity at 183.6 and 182.6 ppm but to a lesser degree compared to the double mutant. In addition, a peak at 182.0 ppm is observed in the K525A mutant at all mole ratios. This peak is not readily observed in wild-type HSA or the R410A/Y411A double mutant at low mole ratios.

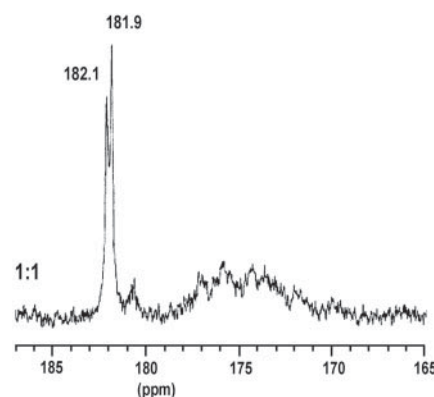


Fig. 4. Titration of isolated domain III of HSA with [^{13}C]palmitate. ^{13}C NMR spectra were obtained to study [^{13}C]palmitate binding to domain III of HSA. Only the carbonyl region of the spectrum is shown (165–187 ppm). Numbers to the left of the spectrum indicate the mole ratio of [^{13}C]palmitate/domain III. Values above the labeled peaks indicate the chemical shift in parts per million. The broad signal centered at ≈ 175 ppm results from protein carbonyl groups. The NMR spectrum for [^{13}C]palmitate bound to domain III revealed only two peaks at 182.1 and 181.9 ppm. These peaks correlate with two of the most intense peaks observed in wild-type HSA and suggest that domain III contains two high-affinity FA-binding sites. Site three is also found in domain III, but stable binding in this site requires electrostatic interactions with residues (R348 and S342) at the edge of domain II.

Efforts to probe binding to FA site three in domain III did not succeed, because the mutant proteins produced (R348A/R485A, R485M, and R348A) suffered from instability problems (data not shown), which may be because of the fact that the substituted side chains all lie at the interface between domains II and III (Fig. 1C) and are involved in stabilizing the tertiary structure. Thus, we assigned the peaks at 182.2 and 181.8 ppm in wild-type HSA to sites four and five, respectively.

The ratio of integrated peak areas from FA and protein carboxyl groups provides a measure of the accumulated binding of FA to the protein. There was a linear relationship between the amount of palmitate added and the FA/HSA carboxyl peak area ratio (Fig. 3). The plotted data are generally similar for palmitate and oleate bound to wild-type HSA, and the calculated values are in agreement with previous studies of [^{13}C]LCFA–albumin complexes (ref. 18; D. P. Cistola and J.A.H., unpublished data). Despite a significant loss of FA binding to one high-affinity site, the total amount of palmitate bound to the R410A/Y411A double mutant was unchanged compared to wild-type HSA. In this case, loss of ^{13}C signal for one peak (182.2 ppm) was compensated by the increase in peak intensities of two other peaks (182.6 and 183.6 ppm). On the other hand, the peak area ratio of the K525A mutant fell below the expected values at all ratios up to 5:1 FA/HSA.

Our assignments of NMR peaks made by the mutations in domain III of full-length HSA then were tested by studying the binding of [^{13}C]palmitate to the recombinant domain III fragment (Fig. 4). This strategy was also expected to simplify the ^{13}C NMR spectrum by removing peaks that originate from binding sites in other domains. Two intense, narrow peaks at 181.9 and 182.1 ppm were observed at a 1:1 ratio. These peaks correspond very closely to two of the major signals that were observed for wild-type HSA at 181.8 and 182.2 ppm, which were linked in the mutagenesis experiments with FA sites five and four, respectively. Both peaks are likely to be associated with high-affinity FA-binding sites, because they are among the first three peaks to appear at low mole ratios of FA/HSA (Fig. 2A). For both wild-type HSA and the domain III construct, the most intense signal occurs at ≈ 181.8 ppm (site five). Therefore, site five likely represents the highest-affinity FA-binding site on the protein. Because only two peaks were

observed in the titration with recombinant domain III, it seems likely that FA site three is unoccupied under our experimental conditions.

Discussion

Binding of ^{13}C -Labeled FA to Albumins. Previous NMR-based studies of the binding of medium-, long-, and very-long-chain FA to BSA and HSA provided important information about several structural features of FA binding, as well as dynamics of the bound FA (18–21, 31, 33–35). Using proteolytic fragments of BSA, ^{13}C NMR identified two high-affinity sites within domain III and one within the N-terminal half of the protein (21). However, additional progress in locating the sites was not possible, because structural information on the binding of FA to BSA at the atomic level was not available.

The crystal structures of FA–HSA complexes revealed the locations of seven binding sites that are common to most types of FA, five of which possess basic or polar side chains that interact closely with the carboxyl group of bound FA and therefore are candidates for designation as high-affinity sites (9–12). However, the saturation methods used to prepare homogenous FA–HSA complexes for crystallization prevented any direct estimate of the relative affinities of the different binding sites. Although the ^{13}C NMR method does not permit absolute affinity measurements, it provides an indication of the relative affinities of different binding sites for the same ligand. Here we exploited the crystallographic information on FA binding to genetically engineered variants or fragments of HSA to extend the reach and precision of NMR spectroscopic methods as a probe of FA–HSA interactions.

We first obtained NMR spectra by titrating [^{13}C]palmitate (C16:0) and [^{13}C]oleate (C18:1) into solutions of wild-type HSA. Both LCFA exhibited a similar pattern of spectral evolution over the course of the titration, although the palmitate spectra were somewhat better resolved. The relative intensities of the different peaks from [^{13}C]palmitate and [^{13}C]oleate bound to HSA were also similar, as were the ratios of integrated FA/protein carboxyl intensities at all FA/HSA mole ratios (Fig. 3). These results show that the binding affinities for both saturated and monounsaturated LCFA at the same binding sites are comparable, which is consistent with predictions of the crystallographic data (11, 12). Given the high degree of homology between BSA and HSA, it is not surprising that the calculated total amount of LCFA bound to HSA closely agrees with previously reported values for LCFA–BSA complexes (8, 18).

Correlation of ^{13}C NMR Chemical Shifts with Specific Binding Sites. A major goal of this study was to establish a methodology for assigning peaks in the NMR spectra obtained for HSA complexed with ^{13}C -labeled FA to binding sites located in the crystal structure, which would allow the identification of the highest-affinity FA-binding sites on the protein (which will correspond to those peaks that appear earliest in the titration) and enhance the value of NMR as a tool for more direct investigation of the binding competition between FA and other HSA ligands in a site-specific fashion. We used two strategies: (i) studies of palmitate binding to HSA with mutations at FA sites four and five in domain III and (ii) studies of palmitate binding to a recombinant wild-type domain III fragment. The NMR data obtained with full-length HSA, even given the improved spectral resolution observed for palmitate/HSA complexes, indicate that the dispersion of chemical shifts associated with each distinct binding site is somewhat limited. The peaks are grouped into a region of the spectrum spanning ≈ 3 ppm and exhibit significant overlap.

Titration of FA into full-length HSA mutants carrying substitutions of FA carboxyl-coordinating residues in sites four and five revealed that the resulting spectra specifically lack resonances at 182.2 and 181.8 ppm, respectively. These are among the three most intense signals observed in wild-type HSA that appear earliest in the titration and therefore represent two of the three highest-affinity FA-binding sites on the protein. This finding is consistent

with previous FA-binding experiments that showed that recombinant domain III of HSA was the only isolated domain that bound myristate (C14:0) (22) and earlier NMR work on a proteolytically generated domain III fragment from BSA, which was shown to retain two high-affinity sites (21). Together, these studies confirm that domain III is the most important domain on albumin for FA binding. Interestingly, there is greater homology between similar domains of albumin from different species than exists among the three domains of albumin from the same species (1), which may partially explain why the distribution of FA sites in HSA is asymmetric and heterogeneous, as opposed to early predictions that FA binding is symmetric throughout albumin (1, 7, 36).

Site II drugs are known to displace FA from site four by interacting with the same residues that are involved in FA binding (37). In one of our preliminary experiments focusing on site II drugs, the addition of diazepam to HSA (5:1 diazepam/HSA) eliminated the peak at 182.2 ppm (data not shown). This perturbation supports the correlation made with the R410A/Y411A double mutant linking this peak to FA site four. In addition, a new peak appeared in the presence of diazepam, with a chemical shift close to the new peak at 182.6 ppm observed in the same mutant when FA were displaced from site four (at a 4:1 FA/HSA ratio). These findings confirm that the double mutation in site four blocked FA binding in this study. Similar studies were also performed by using other site I and II drugs to extend our understanding of the interplay between binding sites; details of these experiments will be reported elsewhere (J.R.S., P.A.Z., S.C., and J.A.H., unpublished data).

Because the peak at 181.8 ppm appears at the lowest mole ratios and remains the most intense peak throughout the titration, site five in subdomain IIIB is most likely the highest-affinity FA-binding site in HSA. This assignment is supported by the finding that nonenzymatic glycosylation of K525 markedly reduces the affinity of HSA for LCFA (38).

To further confirm our peak correlations for both sites and simplify the experimental approach, we probed the FA-binding capacity of a recombinant fragment of HSA that contained only domain III (residues 382–585). As expected, addition of [^{13}C]palmitate to domain III revealed a much simpler spectrum than that obtained with the full-length protein. Moreover, it contained only two well-resolved peaks at 181.9 and 182.1 ppm that corresponded closely to the peaks observed in wild-type HSA at 181.8 and 182.2 ppm. The small chemical-shift difference (0.1 ppm) for each binding site could be caused by minor differences in the tertiary structures of isolated domain III and domain III of full-length HSA. This result confirmed the assignment of peaks within domain III on the basis of the comparative titrations of wild-type and mutant HSA. The finding that the recombinant domain III fragment retains two high-affinity binding sites for FA is in agreement with other studies that have demonstrated that this fragment exhibits wild-type affinity for drugs that are known to bind specifically to drug site II, which is contained within subdomain IIIA (23–25).

Interestingly, a third peak was not observed when FA was titrated into the domain III construct even though this fragment contains most of the structural components of site three (Fig. 4). However, this result is perhaps not surprising given that the carboxyl group of FA molecules bound to site three in intact HSA interact specifically with R348 and S342 at the edge of domain II and with only one residue (R485) in domain III (Fig. 1C) (11, 12). The single remaining electrostatic interaction (with R485) seems to be insufficient to stabilize the FA molecule in site three of the isolated domain. Drugs that bind to drug site II completely overlap with FA site three in subdomain IIIA. Because these drugs bind with essentially wild-type affinity to recombinant domain III (23–25), it seems likely that the hydrophobic cavity of FA site three is preserved in the protein fragment. This finding suggests that domain III preserves the hydrophobic binding pocket of FA site three that normally accommodates the methylene tail of the bound

lipid but that hydrophobic interactions alone are insufficient to stabilize the binding of FA at this site.

FA Redistribution in HSA. For total binding to remain unchanged in the site-four double mutant, FA must be bound elsewhere on the protein. The changes observed in the spectra for the R410A/Y411A mutant at increasing mole ratios support this idea. The increased intensity of the low-intensity peak at 183.6 ppm and the appearance of a new peak at 182.6 ppm at 4:1 could be caused by FA displacement to lower-affinity binding sites and an increase in the ^{13}C NMR signal corresponding to those sites. A similar effect was observed in the K525A mutant. However, the signal at 182.6 ppm did not increase in intensity as much as it did in the site-four mutant. Rather, there was a more intense peak at 182.0 ppm, which was not clearly observed in the other samples. These new peaks at 182.6 and 182.0 ppm could represent two additional low-affinity sites that are not typically observed with wild-type protein under our experimental conditions. It is also possible that these signals could be from FA bound within mutated sites. If substitution of basic residues within each site for nonpolar alanine residues significantly alters the charged microenvironment around the FA carboxyl group, the ^{13}C FA bound to these sites might exhibit a different chemical shift. However, the absence of a peak corresponding to site three in the domain III construct, which also lacks two of the three side chains involved in coordination of the FA carboxyl, suggests that partial loss of these interactions is sufficient to abrogate binding. This hypothesis is supported by other preliminary studies in our laboratory in which similar spectra were observed after displacement of FA from site four with diazepam (data not shown). We believe that the most likely explanation for the enhancement of low-intensity peaks or the appearance of new chemical shifts during titration of FA into mutant HSA is that the lipid is displaced to sites that are not normally evident in spectra of wild-type FA-HSA complexes. In part, these “new” chemical shifts may only have emerged after removal of peaks associated with high-affinity binding that tend to dominate the spectrum.

Integration of the peak area from the FA titration into the site-five mutant (K525A) revealed a significant deficit in total FA

binding when compared to wild-type HSA or the R410A/Y411A double mutant, which is consistent with the finding that nonenzymatic glycosylation of K525 reduces the overall affinity of HSA for LCFA (38) and suggests that displacement of FA to lower-affinity sites is insufficient to compensate for loss of binding to this particular binding site. Although at present there is not an obvious molecular explanation for this observation, it suggests that the loss of site five may significantly impair the ability of HSA to buffer the concentration of FA in plasma. These findings may have implications for FA transport in diabetic individuals and suggest that glucose-mediated impairment of FA binding to HSA may be a complicating factor in the pathophysiology of type two diabetes, which is associated with excess plasma levels of FA (39–41).

Identification and Correlation of Other Binding Sites. Identification of the highest-affinity FA-binding sites by correlating structural and NMR data will permit characterization of FA binding to albumin under changing physiological conditions and allow us to predict likely interactions between competing ligands, especially those that occur at near millimolar concentrations *in vivo*. For example, our current data support earlier suggestions that high-affinity FA binding to site four will likely have an impact on site II drugs, which share a similar binding pocket (11). Our methodology may be extended to complete the assessment of the relative affinities of all FA-binding sites. Once all FA-binding sites have been correlated with specific NMR peaks, our ^{13}C NMR spectroscopic approach will become a powerful tool for solution studies of site-specific analyses of the competition between FA and other ligands (biomolecules and drugs) for HSA. These studies also may give a better understanding of FA transport in the pathological conditions that cause sustained or transient increases in FA/albumin ratios such as diabetic ketoacidosis, myocardial infarction, acute anxiety, and hypoalbuminemia (1, 8).

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