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Quantification of Interactions between Serum Albumin and Endogenous Free Fatty Acids or Exogenous Chemicals by Stable Isotope-Coded Mass Spectrometry

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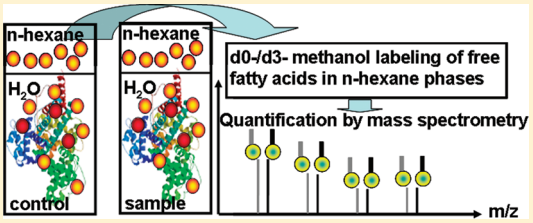
Supporting Information

ABSTRACT: As primary endogenous ligands of serum albumin, free fatty acids exert versatile effects on the albumin conformation through cooperative or competitive interactions with exogenous chemicals. Based on equilibrium partition between *n*-hexane and aqueous phases, we have established three indexes, defined as R_A, R_V, and R_T, for quantitative assessment of the intrinsic binding affinity, the affinitive variation induced by exogenous chemicals, and the topological dependence of albumin affinity, respectively. When albumin molecules in the aqueous phase are in native or denatured forms, or disturbed by exogenous chemicals, corresponding changes of free fatty acids in the *n*-hexane phase can be quantified by an iFFAM (isotope-coded free fatty acid methylation) approach. Free fatty acids from the control and the sample are differentially derived by *d*0- or *d*3-methanol and analyzed by gas chromatography−mass spectrometry. Changes of fatty acids can be revealed by peak ratios of *d*0- or *d*3-labeled fragment ions of fatty acid methyl esters.



KEYWORDS: Mass spectrometry, interactions, serum albumin, free fatty acids, exogenous chemicals

Serum albumin is the most abundant blood protein synthesized in the liver. In addition to the function of maintaining colloid osmotic pressure, serum albumin plays important roles in transporting, distributing, and metabolizing many endogenous or exogenous chemicals.[1] Binding of serum albumin with those compounds determines their plasma concentration and thus subsequent physiological or toxic effects.[2,3] Crystallographic studies[4−7] have shown the structure of this protein in the solid state. However, the three-dimensional structure of serum albumin in aqueous solution and its contribution to binding with various ligands remain largely unknown. Although NMR (nuclear magnetic resonance)[8−10] and fluorescence spectroscopy[11] can provide direct experimental data in solution, the high molecular weight of albumin protein as well as the complication of data interpretation limit their global applications. Recently, with the availability of the genome database, computational modeling based on primary amino acid sequences has emerged as a useful tool to evaluate the binding possibility.[12,13] But every kind of reversible or permanent protein posttranslational modification, such as attachment of small organic groups, cleavage of signal peptides, formation of disulfide bridges, as well as complex unknown physiological status, cannot be predicted from the genome, and it is thus difficult to incorporate these into the computational modeling. The situation is even more difficult for proteins isolated from species that do not have genome databases. Therefore, it is necessary to develop efficient solution-based techniques that can quantitatively describe how the structure of serum albumin determines the interactions with different ligands.

Mass spectrometry-based methods[14] have attracted considerable attention in a broad range of research areas because of their high sensitivity, resolution, and throughput. In this work, the interactions between serum albumin and endogenous free fatty acids as well as exogenous pesticide residues have been investigated by a mass spectrometric approach based on stable isotope-coded free fatty acid methylation (iFFAM). Because free fatty acids are primary endogenous ligands of serum albumin,[15] quantification of variations of unbound free fatty acids in solution under different conditions directly indicates the binding status of serum albumin with exogenous ligands.

The iFFAM approach was based on the equilibrium partition between *n*-hexane and aqueous phases. The principle and major procedures are summarized as follows. In this work, the aqueous solutions containing either native/denatured albumin or the mixture of albumin and exogenous chemicals were incubated with *n*-hexane solutions. Free fatty acids in *n*-hexane phases of the control and the sample were derived with a *d*0- or *d*3-methanol solution of 4% HCl, respectively. The resultant *d*0- and *d*3-fatty acid methyl esters were equally mixed together and analyzed by GC-MS. The original ratios of free fatty acids in the sample over that in the control were recovered by measuring the peak intensities of *d*0- or *d*3-labeled fragment ion pairs. Detailed

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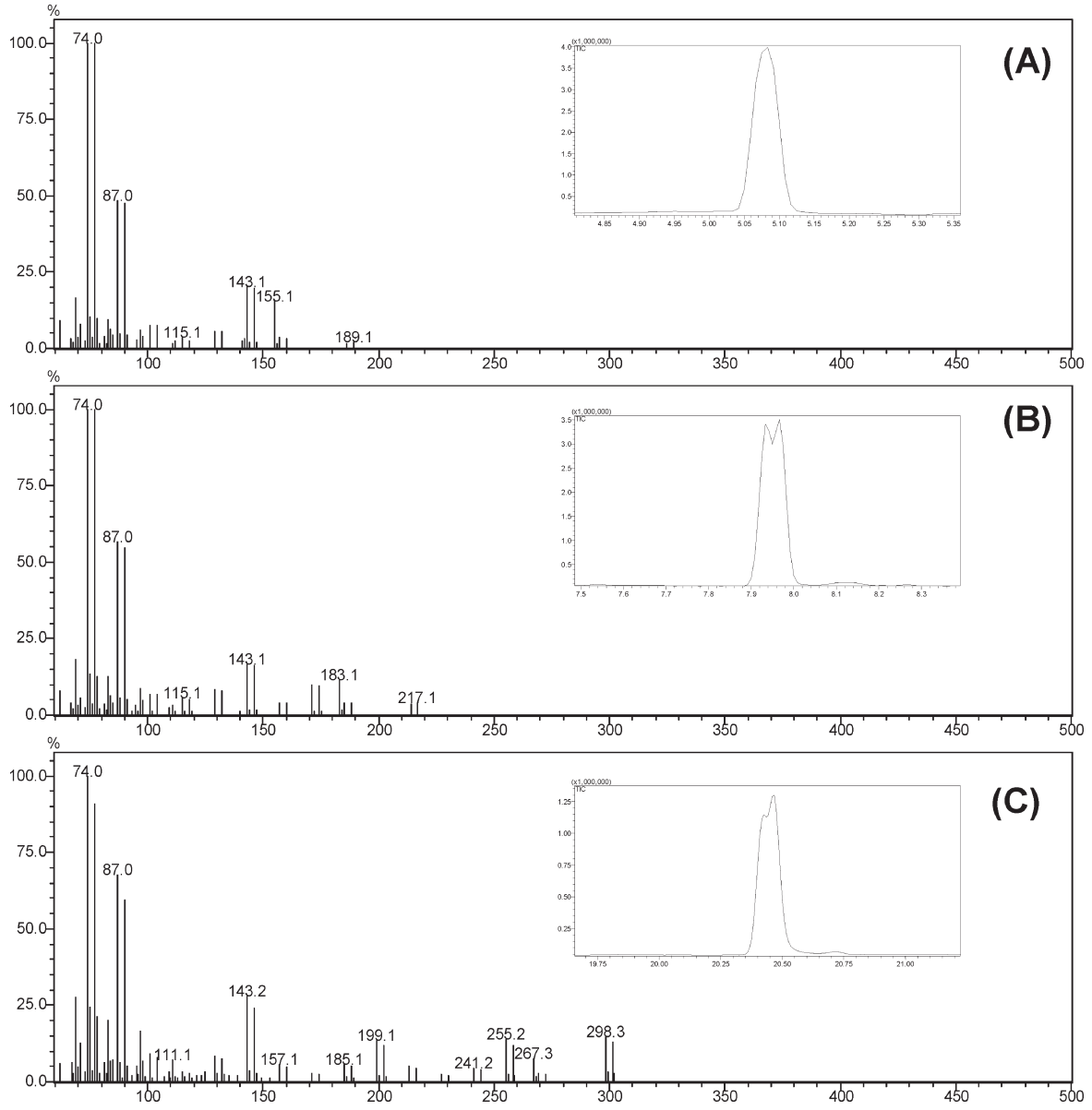

Figure 1. Stable isotope-coded free fatty acid methylation (iFFAM) approach for quantitative analysis of *d*0- or *d*3-methanol derived fatty acid methyl esters at the expected ratio 1.00 (*d*0/*d*3). Parts A, B, and C are averaged mass spectra for d0- and d3-derived C10:0, C12:0, and C18:0 methyl esters, respectively. The insets are enlarged TICs (total ion chromatograms).

experimental procedures and fragment ion pairs used for quantification are listed in the Supporting Information.

In order to evaluate the accuracy and precision of the iFFAM approach, a series of standard free fatty acids, including C10:0, C12:0, C14:0, C16:0, and C18:0, with known concentration were first analyzed according to this procedure. As stated previously,[16] although light and heavy fatty acid methyl esters essentially coelute, there are still a few second differences in retention time due to the high separative capacity of the 30 m long capillary chromatographic column. In order to minimize the isotope effect, all scans were selected and automatically averaged by clicking-and-dragging the cursor across the whole elution window. The insets of parts A, B, and C of Figure 1 are enlarged TICs (total ion chromatograms) of the C10:0, 12:0, and C18:0 methyl esters, respectively. It was found that there is less isotope effect for C10:0 than that for C12:0 and C18:0. Figure 1 shows the averaged mass spectra generated from all scans within each elution window. It is shown there are several pairs of fragment ions. Relative quantities are determined by averaging the peak ratios of *d*0- or *d*3-labeled fragment ion pairs (m/z = 74/77, 87/90, 143/146, m/z = 74/77, 87/90, 143/146, 171/174, and m/z = 74/77, 87/90, 143/146, 199/202, 255/258, 298/301 for C10:0, C12:0, and C18:0 methyl esters, respectively). The observed average peak ratios (d0:d3) are 1.01 ± 0.05, 1.04 ± 0.03, and 1.04 ± 0.05 for these three fatty acid methyl esters (when the expected ratio is 1.00). Supporting Information Figure 1 shows the mass spectra of d0-and d3-labeled C10:0 methyl esters at the expected ratios of 0.50, 0.33, 0.25, and 0.20 (*d*0/*d*3), respectively. It was demonstrated that this isotope-coded mass spectrometric approach can quantitatively detect changes of unbound free fatty acid in solution. For example, the observed mean ratios for the C10:0 methyl ester are 1.01, 0.55, 0.34, 0.26, and 0.20 when the

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expected ratios are 1.00, 0.50, 0.33, 0.25, and 0.20, respectively ($d0/d3$). All data of five standard fatty acid methyl esters mixed at different known quantities was summarized in Supporting Information Table 1. The mean differences between observed and expected quantities for the five standard free fatty acids ranged from 0% to 22%, and the standard deviations ranged from 0 to 0.08. The quantitative measurement of differentially labeled free fatty acids in the control and in the sample provides a means to monitor the dynamic status of albumin−fatty acid complexes in response to different treatments.

It is known that the affinity of albumin is dynamic and complicated.[17] Investigations of the versatile binding properties of albumin in aqueous solution have been hampered due to a lack of a suitable method that can quantify the complexity and flexibility of the protein affinity toward different ligands. The iFFAM approach provides a nonradioactive labeling means to quantify unbound free fatty acids. Changes in the level of unbound free fatty acids straightforwardly reflect the changes of albumin affinity induced by cooperative/competitive binding with exogenous chemicals or by denaturation of the folded native structure. Herein, essential fat free goat albumin (GSA) was used as a model. When native GSA solution or blank buffer solution was incubated with *n*-hexane solutions containing a known amount of free fatty acids, the quantities of free fatty acids (RCOOH) remaining in *n*-hexane layers were related with several equilibria (Supporting Information), including a partition equilibrium between the aqueous phase and the *n*-hexane phase, a dissociation equilibrium in the aqueous phase, as well as a stepwise formation equilibrium of albumin−fatty acid complexes in the aqueous phase. As described previously, unbound free fatty acids remaining in the *n*-hexane phases that have been incubated with blank or buffered GSA solution can be differentially derived by *d*0- or *d*3-methanol, respectively. The ratio of *d*0- or *d*3-labeled fatty acid methyl esters is defined as the intrinsic index R_A of albumin affinity toward different free fatty acids.

$$R_A = \frac{[\mathrm{RCOOH}]_{\mathrm{blank}}(\mathrm{d0-methylester})}{[\mathrm{RCOOH}]_{\mathrm{GSA}}(d3-\mathrm{methylester})} \quad (1)$$

Higher R_A indicates less free fatty acids in the *n*-hexane phase that has been incubated with native GSA solution, meaning higher intrinsic affinities of albumin toward fatty acid anions. The minimum value of R_A is 1, which means the amount of free fatty acids remaining in *n*-hexane phases that has been incubated with blank buffer solution or buffered GSA solution is the same and albumin protein has the lowest intrinsic affinity toward a specific fatty acid. Because the binding ability of albumin in solution was affected by lots of unpredicted factors, such as the unknown physiological status or the unknown effects of experimental conditions, the proposed stable isotope labeling strategy can provide an internal reference for quantitatively delineating the complex interactions among different molecules.

Averaged peak ratios of *d*0- and *d*3-methanol derived fatty acid methyl esters and standard deviations were summarized in Table 1. Supporting Information Figure 2 shows representative mass spectra. The intrinsic indexes R_A for C10:0, C12:0, C14:0, C16:0, and C18:0 are 2.40, 3.96, 2.33, 1.47, and 1.30, respectively. It can be found that medium chain fatty acids (C10:0, C12:0, and C14:0) have different binding properties compared with long chain fatty acids (C16:0 and C18:0). In order to assess if there is cooperative or competitive binding among these five fatty acids, these fatty acids have been mixed together in *n*-hexane solution but with the same concentration as that used in the above experiments. Then *n*-hexane solutions containing the mixture of all five free fatty acids were incubated with buffered GSA solution or blank buffer solution. Supporting Information Figure 3 shows representative mass spectra of the five fatty acid methyl esters ($d0/d3$) remaining in the *n*-hexane layers, and the experimental data was summarized in Table 1. It was shown that the affinity index R_A for all these fatty acids decreased, indicating that these fatty acids compete with each other to bind with albumin.

Table 1. Intrinsic Index R_A of Albumin Affinity toward Free Fatty Acids

	mean ± SD (blank/GSA)	
FAMEs[a]	partition with single fatty acids	partition with a mixture of fatty acids
C10:0	2.40 ± 0.80	1.59 ± 0.26
C12:0	3.96 ± 0.83	2.00 ± 0.11
C14:0	2.33 ± 0.35	1.40 ± 0.18
C16:0	1.47 ± 0.61	0.97 ± 0.10
C18:0	1.30 ± 0.55	1.01 ± 0.15

[a] Fatty acid methyl esters.

The R_V index is aimed to quantify how endogenous free fatty acids impact the binding of albumin with exogenous chemicals. As shown in eq 2, it is based on the quantitative comparison of unbound free fatty acids in *n*-hexane phases when the aqueous solution of albumin−fatty acids complexes was spiked or not spiked with exogenous chemicals. Fat-free goat serum albumin (GSA) solution was incubated with the mixture of free fatty acids C10:0, C12:0, C14:0, C16:0, and C18:0 at a molar ratio of 3 (each fatty acid/albumin) in a 37 °C shaker for 12 h to obtain albumin−fatty acid complexes. And then DDT (4,4′-dichlorodiphenyltrichloroethane, an organochlorine pesticide) or ethion (*O*,*O*,*O*′,*O*′-tetraethyl *S*,*S*′-methylene di(phosphorothiolothionate), an organophosphate pesticide) was added to the solution at a molar ratio of 10 (DDT or ethion/each fatty acid) for another 12 h of incubation at 37 °C with shaking. The solution without spiking DDT or ethion was used as the control. Unbound free fatty acids from the control or from the sample were subsequently extracted with *n*-hexane, derived by *d*3- or *d*0-methanol, respectively, and analyzed by the iFFAM approach. Supporting Information Figure 4 lists representative mass spectra. Induced by exogenous chemicals, the variable index R_V of the albumin affinity toward different endogenous fatty acids was defined as the intensity ratio of fragment ion pairs of *d*0- and *d*3-derived fatty acid methyl esters ($d0/d3$).

$$R_V = \frac{[\mathrm{RCOOH}]_{\mathrm{spiking}}(d0-\mathrm{methylester})}{[\mathrm{RCOOH}]_{\mathrm{no\text{-}spiking}}(d3-\mathrm{methylester})} \quad (2)$$

If exogenous chemicals do not impact the albumin affinity toward endogenous free fatty acids, the value of the R_V index is 1. When the R_V value is less than 1, the smaller the R_V index value, the stronger the enhancement of exogenous chemicals on albumin affinity toward endogenous free fatty acids. When the R_V value is more than 1, the larger the R_V index value, the stronger the reduction of exogenous chemicals on albumin affinity toward endogenous free fatty acids. As shown in Table 2, when DDT was spiked in the solution, the variable index R_V

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Table 2. Variable Index R_V of Albumin Affinity toward Free Fatty Acids Induced by Exogenous DDT and Ethion

	R_V(spiked with DDT)	R_V(spiked with ethion)
FAMEs[a]	mean ± SD (DDT spiked/nonspiked)	mean ± SD (ethion spiked/nonspiked)
C10:0	1.03 ± 0.11	1.03 ± 0.05
C12:0	1.05 ± 0.09	1.08 ± 0.07
C14:0	0.97 ± 0.07	1.00 ± 0.14
C16:0	0.69 ± 0.11	0.68 ± 0.13
C18:0	0.79 ± 0.14	0.97 ± 0.22

[a] Fatty acid methyl ester.

Table 3. Topological Index R_T of Albumin Affinity toward Free Fatty Acids

	mean ± SD (denatured/native)	
FAMEs[a]	R_T(denatured by HCl)	R_T(denatured by tryptic digestion)
C10:0	6.60 ± 0.11	>100
C12:0	2.70 ± 0.08	>100
C14:0	1.67 ± 0.04	>100
C16:0	0.97 ± 0.05	6.02 ± 0.91
C18:0	0.96 ± 0.09	5.79 ± 0.98

[a] Fatty acid methyl ester.

values of albumin affinity for the medium chain fatty acids C10:0, C12:0, and C14:0 are around 1, meaning exogenous DDT does not significantly impact the affinity of albumin toward these endogenous fatty acids. However, the R_V values for C16:0 and C18:0 are less than 1, indicating the presence of observable enhancement of albumin affinity toward these two fatty acids. When ethion was spiked in the solution, the variable index R_V values of albumin affinity for C10:0, C12:0, C14:0, and C18:0 are around 1. But only the R_V for C16:0 is less than 1, indicating the interaction of albumin with ethion enhances albumin affinity toward C16:0 fatty acid.

Although crystallographic analysis has revealed that different binding sites on albumin protein display distinct topological differences, including flat slots, narrow tunnels, or surface trenches,[1−7,17−19] the effects of these different pockets on entrapping endogenous and exogenous ligands in solution remains unknown. As demonstrated previously, the intrinsic indexes R_A of serum albumin for medium chain fatty acids (C10:0, C12:0, and C14:0) are higher than that for long chain fatty acids (C16:0 and C18:0). This means that binding of fatty acids to albumin is not a simple function of hydrophobic chain length. This experimental result is also in accordance with the evidence shown in the section about the variation of albumin affinity induced by exogenous chemicals. It was shown that the binding of these medium chain free fatty acids with albumin is much more resistant to spiked exogenous chemicals.

To investigate the topological dependence of albumin affinity, differences in unbound free fatty acids between the native and denatured solutions of goat albumin−fatty acid complexes (albumin/fatty acid = 1:3) were analyzed by the iFFAM approach. The topological index R_T of albumin affinity was defined as the intensity ratio of fragment ion pairs of *d*0- or *d*3-derived fatty acid methyl esters obtained from the denatured solution over that from the native solution (as shown in eq 3).

$$R_T = \frac{[\mathrm{RCOOH}]_{\mathrm{denatured}}(d0\text{-methylester})}{[\mathrm{RCOOH}]_{\mathrm{native}}(d3\text{-methylester})} \qquad (3)$$

Higher R_T means higher topological dependence of the binding of free fatty acids with albumin. The minimum R_T is 1, meaning no observable topological dependence. In order to disrupt the folded native structure of goat albumin, the solution of albumin−fatty acid complexes (pH 7.4 in Krebs−Ringer buffer) was reduced by DTT, carbamidomethylated by iodoacetamide, and digested by trypsin at 37 °C overnight, or adjusted to pH 2 by using 0.1 M HCl. Representative mass spectra were shown in Supporting Information Figure 5, where the native structure of albumin was partially destroyed by 0.1 M HCl (pH 2), or in Supporting Information Figure 6, where the native structure of albumin was completely destroyed by trypsin digestion. All experimental data was summarized in Table 3. It can be found that the topological indexes R_T of albumin for medium chain fatty acids (C10:0−C14:0) are more than 1 when the native structure of serum albumin is partially destroyed by 0.1 M HCl. In contrast, the R_T indexes for long chain fatty acids (C16:0 and C18:0) are around 1, meaning there is no observable topological dependence. Moreover, when the native structure of albumin is completely disrupted by trypsin digestion, the R_T indexes for medium chain fatty acids (C10:0−C14:0) are substantially increased to more than 100. But the R_T value for long chain fatty acids (C16:0 and C 18:0) are only increased to 6.02 and 5.79, respectively. The experimental results indicate that free fatty acids with smaller size cannot be efficiently extracted by *n*-hexane until the folded nature structure is disrupted. The binding affinities of albumin toward different free fatty acids are not solely dependent on the hydrophobic effects. The topological differences in different binding sites play critical roles in entrapping and absorbing different ligands.

By using this approach, we have studied the composition of fatty acids in nondefatted bovine serum albumin. Bovine serum albumin was dissolved in the same Krebs−Ringer buffer solution (pH 7.4) as that used in the above experiments. HCl and trypsin digestion were used for denaturation too. Nondenatured and denatured bovine serum albumin solutions (15 μg/μL) were incubated with *n*-hexane respectively. The resultant fatty acids present in *n*-hexane layers were analyzed by the iFFAM approach. As shown in Supporting Information Table 2, the recoveries of medium chain fatty acids C10:0 and C12:0 from denatured albumin were substantially increased. But there was no significant improvement in the recoveries of C14:0, C16:0, and C18:0. Supporting Information Figure 7 (I−V) and (A−E) show representative mass spectra. These experiments clearly indicate that medium chain fatty acids were much more tightly entrapped in the binding pockets than long chain fatty acids. The binding affinity is not a simple function of hydrophobic chain length.

Early in 1972,[20] Tanford found that the free energies of binding were less than that expected for ligands having a chain length of more than eight carbon atoms, although they were linearly dependent on hydrocarbon chain length for ligands having shorter chain length. It was assumed that the size limitations of the binding sites might play important roles in the albumin affinity. Later, Ashbrook[21] obtained similar results and explained this observation by the configurational adaptability of albumin binding sites as fatty acids increase in length. Ulrich[22]

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indicated the presence of a different mechanism instead of a simple hydrophobic interaction, i.e. fatty acid binding to different regions of the albumin molecule. High-resolution crystallographic analysis of human serum albumin[5] provides further evidence that different binding sites have different topological properties. Topological differences in pockets such as flattened slots, narrow tunnels, and surface trenches result in distinct binding affinities for different ligands. For example, the narrow tunnel of site 2 located between subdomain IA and IIA is one of the most enclosed fatty acid binding sites and strongly shielded from solvents. Binding at this site has been considered to be able to drive the conformational changes. Fatty acids including C10:0, C12:0, C14:0, C16:0, and C18:0 are all found at this site. In particular, the electronic density map clearly indicates the presence of the second fatty acid binding at this site for medium chain fatty acids but no density at all for a second fatty acid longer than C14:0. This result indicates that medium chain fatty acids might extend further into the cavity so there is enough room for a second binding. In summary, because serum albumin performs its function in aqueous solution, quantitative approaches based on aqueous solution are important to facilitate a better understanding of how this high abundance plasma protein interacts with endogenous and exogenous molecules. The iFFAM approach quantitatively assesses the albumin affinity by three indexes: (1) R_A was determined by the intrinsic properties of ligands and proteins; (2) R_V was determined by conformational variations of albumin caused by exogenous ligands; and (3) R_T was determined by topological properties of binding sites. It should be applicable not only to serum albumin−fatty acid interactions but also to global protein−fatty acid interactions that are of interest in pharmaceutical research, environmental health, and biological science.

■ ASSOCIATED CONTENT

Supporting Information. Information on reagents, instrument setup, experimental procedures, and detailed tables/figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Tingting Li performed the experiments. Yingxia Yue, Jianjian Li, Xiaoli Wang, and Jieying Fu were involved in the lab work. Hongying Zhong raised the original concept, designed the experiments, and wrote the manuscript.

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