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A Molecular Mechanism for Modulating Plasma Zn Speciation by Fatty Acids

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Supporting Information

ABSTRACT: Albumin transports both fatty acids and zinc in plasma. Competitive binding studied by isothermal titration calorimetry revealed that physiologically relevant levels of fatty acids modulate the Zn-binding capacity of albumin, with far-reaching implications for biological zinc speciation. The molecular mechanism for this effect is likely due to a large conformational change elicited by fatty acid binding to a high-affinity interdomain site that disrupts at least one Zn site. Albumin may be a molecular device to "translate" certain aspects of the organismal energy state into global zinc signals.

We present evidence for a fatty-acid-mediated reduction in the Zn-binding ability of serum albumin. This provides a direct molecular link between fatty acid metabolism and the plasma Zn distribution.

A plethora of biological pathways and signaling cascades are directly affected by zinc,[1] and many disease states, including neurodegenerative and cardiovascular diseases, diabetes mellitus, asthma, and cancer, are accompanied by systemic zinc dyshomeostasis.[2,3] However, in the majority of cases, the underlying molecular mechanisms remain unknown. One important area of interest concerns the links between zinc homeostasis and energy metabolism.[4] Besides the well-established link between zinc and insulin,[5] extensive phenomenological data at the organismal and cellular levels are available for other pathways influenced by zinc; for example, it is well-known that zinc affects appetite[6] and that the synthesis of fatty acids and their esterification in adipocytes (lipogenesis) is zinc-induced.[7] Thus, zinc clearly has a multifaceted regulatory/signaling role in fat metabolism.

Understanding the regulatory roles of zinc in a biological system requires an understanding of Zn-trafficking mechanisms. How does the appropriate amount of Zn reach the appropriate cells in a healthy individual? How does Zn dyshomeostasis occur, and how does this affect metabolic processes? Much recent progress has been made with the identification and study of the many membrane-bound Zn transporters of the ZIP and ZnT families.[3,8] Another important checkpoint in the Zn homeostatic system appears to be the blood plasma. About 75% of total Zn (15−20 μM[9]) is bound to serum albumin,[10] the most abundant plasma protein (ca. 600 μM),[11] which contains 585 amino acids arranged into three homologous domains (Figure 1). One of its major functions is the transport and delivery of fatty acids, which otherwise are only sparingly soluble in aqueous solution. Crystallographic studies have identified five major and up to five additional low-affinity binding sites for fatty acids with different chain lengths.[12] Importantly, rather than being an indiscriminate sponge for a variety of molecules, albumin is a biologically active protein with regulatory functions for many cell types.[13]

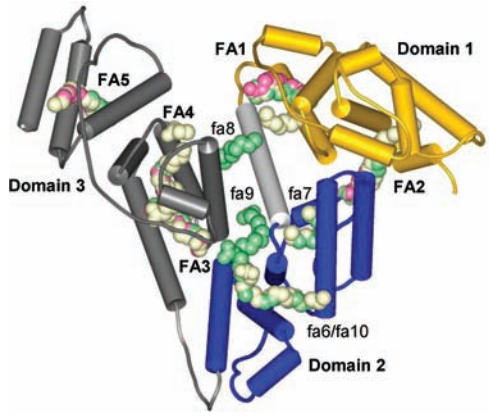

Figure 1. Domain structure of albumin and fatty acid binding sites. Overlaid structures with PDB codes: 1bj5, HSA with five myristates, pink (the protein backbone is also shown); 1e7e, HSA with 10 decanoates, green; 1gnj, HSA with seven arachidonates, light-yellow.[12] FA1−5, major sites; fa6−10, minor sites.

Our investigation was based on the recent structural characterization of the major Zn binding site A on human serum albumin (HSA; Figures 2A and 4B).[14] This site bridges the domain I and II interface and is formed by His67 and Asn99 in domain I and His247 and Asp249 in domain II. X-ray crystal structures of HSA revealed that the site is disrupted in all fatty-acid-bound structures [Figure 2A and Figure S1 in the Supporting Information (SI)] as a result of a large conformational change induced by fatty acid binding to site FA2. This site is composed of two half-sites, one in each of domains I and II. A fatty acid in this site acts like a pin that fixes the two

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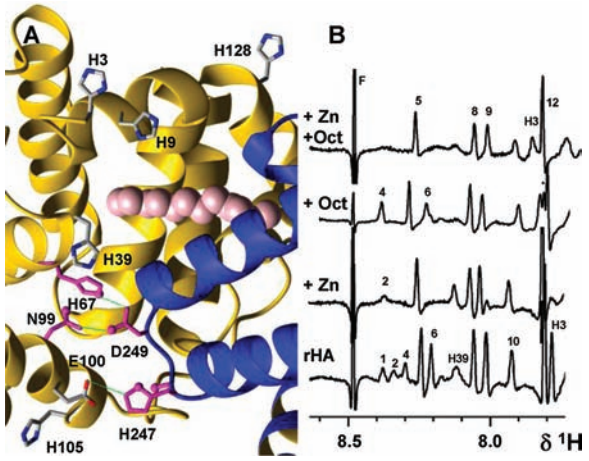

Figure 2. Binding events at the domain I/II interface affect several His Hε1 resonances. (A) Interface with the disrupted Zn-binding site A (magenta) and interdomain His residues likely to be affected by fatty acid binding in site FA2. The 11 resolved carbons of Myr2 in PDB entry 1bj5 are shown as pink spheres. (B) Effects of 1 molar equiv of Zn and 5 molar equiv of OCT on His Hε1 resonances. Peaks 1 and 4 are assigned to His67 and His247;[14] full titration data are shown in Figure S3.

domains in an orientation that differs significantly from the fatty-acid-free conformation.[12] The physiological relevance of this dramatic conformational change has remained enigmatic.

We hypothesized that simultaneous zinc binding to site A and fatty acid binding to site FA2 may be incompatible.[14] This was supported by the observation that high concentrations of either natural fatty acids or octanoate (OCT; C8) lead to the perturbation of a peak corresponding to site A in ^{111}Cd or ^{113}Cd NMR spectra (Figure S2).[14,15] However, it has not been demonstrated experimentally whether Zn^{2+}, the actual physiological binding partner, hampers fatty acid binding to albumin or vice versa. Neither Zn^{2+} nor fatty acids possess readily exploitable spectroscopic features, but ^{1}H NMR spectroscopy has previously been used to study protonation equilibria, conformational changes, and Zn^{2+} binding via monitoring of histidine Hε1 resonances.[14,16] We used this method to explore the effects of OCT on Zn^{2+} binding to albumin.

Addition of OCT to HSA (Figure 2B and Figure S3) had a strong effect on several resonances, including those of the two Zn-binding His residues (peaks 1 and 4[14]), consistent with a significant influence of fatty acid binding on the environment of these residues and with the perturbation of peak A in ^{111}Cd NMR spectra. However, peak 4 was absent in the presence of both Zn^{2+} and OCT, just as in the presence of Zn^{2+} alone. This suggests that the presence of OCT does not abolish Zn binding to site A, despite its clear effect on ^{111}Cd binding. Thus, although ^{1}H NMR allowed these separate binding events to be monitored and confirmed the participation of His67 and His247 in both cases, it could not resolve whether Zn binding is thermodynamically favored over OCT binding or whether simultaneous binding of OCT to FA2 and Zn^{2+} to site A is possible.

To address this, we developed an isothermal titration calorimetry (ITC) approach to study competitive binding. ITC is universally applicable to equilibrium reactions, as it measures thermal effects arising from molecular interactions, and it has been used successfully for the determination of metal–protein stability constants,[17] including those of Cu, Ni, and Co with albumin.[18] Interactions between proteins and fatty acids have also been studied by microcalorimetry,[19] but no calorimetric studies of competitive metal/fatty acid binding to a protein have been reported.

First, binding to bovine serum albumin (BSA) in Tris-buffered solutions at pH 7.2 was studied. BSA was chosen because of the high sample consumption of ITC; notably, however, the sequences of BSA and HSA are 75% identical, and their binding properties for Zn and fatty acids are very similar.[11,20,21] The reaction of Zn^{2+} with BSA under these conditions was exothermic (Figure S4), and in agreement with literature findings,[20,21] more than 1 molar equiv of Zn^{2+} could bind to BSA. Under the experimental conditions, two binding constants were captured (Figure 3A). Previous equilibrium

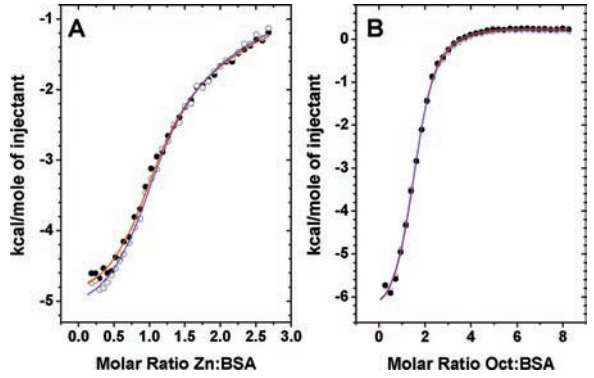

Figure 3. Competitive binding of Zn and OCT to BSA studied by ITC. (A) Zn binding to BSA (25 μM) in the presence and absence of 5 molar equiv of OCT. (B) OCT binding to BSA and Zn_1BSA. Open circles with blue fits correspond to binary systems and filled circles with red fits to ternary systems. In each case, 34 injections of 8 μL of 333 μM ligand (Zn or OCT) were delivered over 16 s with 240 s between injections for complete equilibration.

dialysis studies suggested the presence of a third Zn binding site,[21] but it was too weak to be detected at the albumin concentration employed. Evaluation of the data using a model with two sequential binding constants yielded a conditional stability constant, log K_{ZnBSA}' = 5.67, for the first equivalent of Zn^{2+}. Correction for the effects of pH and Tris concentration (see the SI) gave log K = 7.0 ± 0.3 for the stoichiometric constant, in reasonable agreement with literature values.[20,21]

ITC was also applied to study fatty acid binding to albumin. Several data sets for OCT binding at various albumin concentrations (25, 50, and 500 μM) were acquired (Figure 3B and Figures S5 and S6). Fitting models employing two sets of binding sites gave log K = 5.4 ± 0.4 for the highest-affinity class and log K = 3.3 ± 0.4 for the other set of sites. Previous studies of OCT binding reported values between 6.3 (Scatchard plots from rate-of-dialysis measurements)[22] and 4.53 (stepwise constants from equilibrium dialysis measurements)[23] for the highest-affinity site.

After it had been established that ITC yields thermodynamic data consistent with literature values for the binary systems, the ternary system was studied to investigate whether Zn^{2+} and OCT binding to BSA are interactive. The results of titrations with Zn^{2+} in the presence of 5 molar equiv of OCT (to ensure that FA2 was populated) were indistinguishable from those in the absence of fatty acid (Figure 3A). Conversely, the presence of 1 molar equiv of Zn^{2+} did not significantly affect the affinity

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or stoichiometry of OCT binding to BSA at any of the concentrations studied (Figure 3B and Figure S6).

Thus, the ^{1}H NMR data for the ternary system likely reflect simultaneous binding of Zn and OCT. Bhattacharya et al.[12b] reported that HSA in the presence of OCT does not crystallize in a form that is isomorphous with all other fatty-acid-containing X-ray crystal structures, and they speculated that at least 10 carbon atoms may be needed to elicit the fatty-acid-induced conformational change. Our molecular model in which both Zn and OCT are bound simultaneously (Figure 4B)

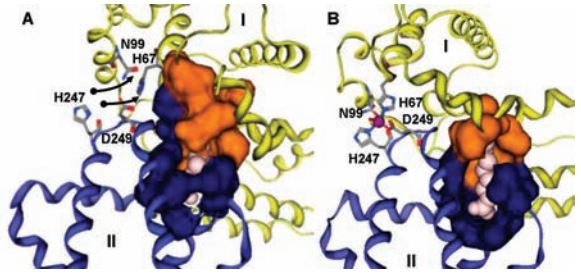

Figure 4. Different binding modes for (A) medium- and (B) short-chain fatty acids in site FA2 on HSA. Fatty acid molecules are shown in pink. The colored surfaces represent Analytical Connolly surfaces of the residues forming the binding pocket. In both models, the carboxylate headgroup interacts with R257, and the hydrophobic half-pocket in domain II (blue) is formed by residues L250, L251, A254, A258, L283, and L284. (A) HSA with bound MYR, based on PDB entry 1bj5. Three C atoms have been added to the C11 chain resolved in the X-ray structure. Domain I (orange and yellow) contributes to the fatty acid binding site an extended half-pocket comprising residues R10, L14, F19, L22, V23, A26, L66, and Y150. The complete pocket can be formed only if the zinc site (labeled residues) is disrupted. (B) HSA with OCT and Zn^{2+} (purple) bound simultaneously. OCT is short enough to be accommodated predominantly in the domain II pocket. Hydrophobic residues L14, F19, L22, and L155 form a new half-pocket without disrupting the zinc site.

shows that a C8 chain can indeed be accommodated in a truncated FA2 site without the conformational change observed upon binding of longer-chain fatty acids.

To address the suspected impact of chain length, we conducted further competition experiments using the C14 fatty acid myristate (MYR). The binding of MYR to albumin (Figure S7) closely matches that of the physiologically most abundant palmitate and stearate in terms of binding sites[12] but is slightly weaker.[23] Titrations with Zn^{2+} in the presence of increasing amounts of MYR (Figure 5A) revealed that the stoichiometry (Figure 5B) and/or affinity of Zn^{2+} decrease dramatically in the presence of >1 molar equiv of MYR. Conversely, MYR titrations of the 1:1 Zn:BSA complex showed that the energetics but not the stoichiometry of the binding reaction are affected by Zn^{2+}, as indicated by a decrease in affinity and exothermicity (Figure 5C; $\Delta\Delta H$ = 1.1 kcal/mol, average for five Myr). These observations can be rationalized by assuming that the binding of MYR requires the dissociation of Zn^{2+} from BSA; since the binding reaction is exothermic (ΔH = −4.7 kcal/mol), this dissociation must be endothermic, although the difference in experimental conditions precludes direct quantitative comparisons.

Besides highlighting the complexity of a system with two or three binding sites for one ligand and 5−10 binding sites for another, these experiments unequivocally confirm the hypothesis that binding of long-chain fatty acids to albumin and Zn^{2+}

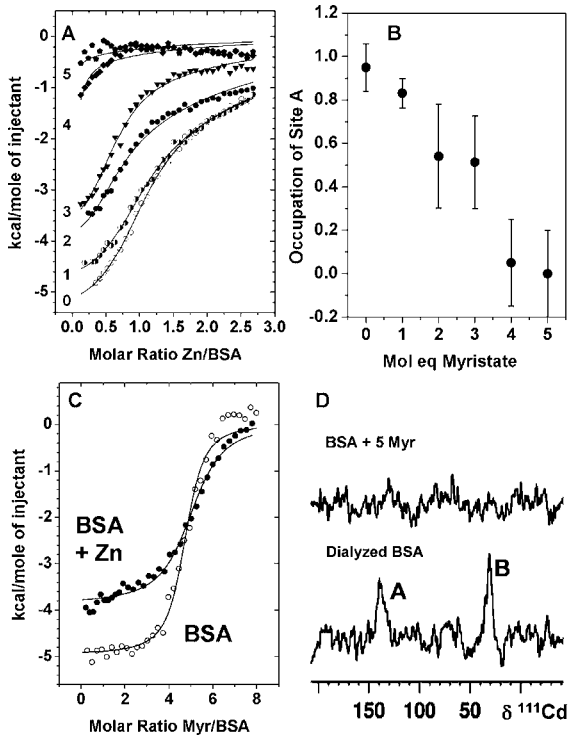

Figure 5. Competitive binding of metals and MYR to BSA. (A) Effect of increasing amounts of MYR on the zinc-binding capacity of BSA. ITC curves for titrations of 333 μM Zn^{2+} into 25 μM BSA in the presence and absence of varying amounts (0−5 molar equiv) of MYR in 50 mM Tris/50 mM NaCl (pH 7.2). The fits (Figure S8) allowed estimates of the ratio of site A availability, as shown in (B). A clear downward trend was observed. A 4:1 MYR:Zn molar ratio suppressed occupation of site A almost completely. A second binding site was also affected by fatty acid binding (see D). (C) ITC curves for titrations of 500 μM MYR into 12.5 μM BSA or Zn_1BSA. These titrations were carried out in H_2O because of the insufficient solubility of MYR in Tris buffer. The fits (Figure S9) correspond to a model with one set of binding sites to estimate the stoichiometry for the highest-affinity sites. This equaled 5.0 ± 0.3, and the average log K was 6.3 ± 0.4 in the absence and 5.9 ± 0.4 in the presence of Zn^{2+}. More complex fits were possible, but the data were insufficient to justify them. (D) ^{111}Cd NMR spectra of Cd_2BSA recorded in the absence and presence of 5 molar equiv of MYR. Peaks A and B were both suppressed by MYR.

is interactive. They also indicate that the affinity of MYR is higher than that of Zn^{2+} and suggest that elevated levels of physiological fatty acids have a striking effect on Zn binding to albumin. Inhibition of the binding to HSA of a thiosemicarbazonato complex of Cu^{2+} by stearate was reported,[24] but no molecular explanation was given. Unexpectedly, a second Zn-binding site was also affected by MYR (Figure 5A). The location of this site is unknown, but a likely candidate is Cd site B. Indeed, addition of 5 molar equiv of MYR to Cd_2BSA perturbed both peak A and peak B (Figure 5D). An effect of fatty acids on site B has not been reported previously.

Our observations raise new questions regarding plasma Zn distribution and its dependence on fatty acid levels and stress the need to identify site B. Under normal physiological conditions, 0.1−2 fatty acid molecules are bound to albumin. The allosteric switch we have studied may play a so-far overlooked role in fatty-acid-mediated Zn fluxes and explain marked shifts in the systemic Zn distribution[2−5] under a variety of conditions that are also characterized by high plasma levels of fatty acids,[11,25−27] such as fasting, exercise, and pathological

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states such as obesity,[28] diabetes, and liver or cardiovascular disease, including atherosclerosis[28] and myocardial infarction.[29] Besides the major effects expected at abnormally high levels of fatty acids, however, possibly the most important outcome of this study is the fact that we observed a measurable effect even with just 1 or 2 molar equiv of fatty acid (i.e., normal physiological levels). This is consistent with conclusions by Simard et al.,[26] who identified FA2 as one of the three high-affinity sites on HSA. Importantly, the ^{13}C NMR peak corresponding to palmitate bound to FA2 began to emerge even at a ratio of 1:1.[27] This suggests that the affinities of FA sites 4 and 5 (known high-affinity sites) and 2 are very similar and that FA2 is indeed populated to a considerable extent even at normal physiological fatty acid levels. Together with the present study, this means that fatty acids at all concentrations have an impact on the distribution of Zn^{2+} in plasma.

The direct consequences of Zn displacement from albumin are unknown, but because Zn is both a signaling agent and potentially toxic to cells,[14,30] significant effects can be expected even if just a fraction of plasma Zn becomes mobilized by subtle changes in plasma fatty acids. This molecular link between energy metabolism and Zn speciation may prove to be of clinical significance. Among the wide range of metabolic processes affected by zinc,[1] we highlight documented plasma zinc effects on cytokine biology[31] and hemostasis,[32] including blood coagulation. This may be mediated by the Zn-dependent interaction between histidine-rich glycoprotein and the anticoagulants heparin and heparan sulfate.[33]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details, complete ref 4b, site A residues in published X-ray structures, and NMR and ITC data. This material is available free of charge via the Internet at http://pubs.acs.org.

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