Novel molecular interactions of acylcarnitines and fatty acids with myoglobin

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ABSTRACT

Previous research has indicated that long-chain fatty acids can bind myoglobin (Mb) in an oxygen dependent manner. This suggests that Oxy-Mb may play an important role in fuel delivery in Mb-rich muscle fibers (e.g., type I fibers and cardiomyocytes), and raises the possibility that Mb also serves as an acylcarnitine binding protein. We report for the first time the putative interaction and affinity characteristics for different chain lengths of both fatty acids and acylcarnitines with Oxy-Mb using molecular dynamic simulations and isothermal titration calorimetry experiments. We found that short- to medium-chain fatty acids or acylcarnitines (ranging from C2:0 to C10:0) fail to achieve a stable conformation with Oxy-Mb. Furthermore, our results indicate that C12:0 is the minimum chain length essential for stable binding of either fatty acids or acylcarnitines with Oxy-Mb. Importantly, the empirical lipid binding studies were consistent with structural modeling. These results reveal that: (i) the lipid binding affinity for Oxy-Mb increases as the chain length increases (i.e., C12:0 to C18:1), (ii) the binding affinities of acylcarnitines are higher when compared to their respective fatty acid counterpart, and (iii) both fatty acids and acylcarnitines bind to Oxy-Mb in 1:1 stoichiometry. Taken together, our results support a model in which Oxy-Mb is a novel regulator of long-chain acylcarnitine and fatty acid pools in Mb-rich tissues. This has important implications for physiological fuel management during exercise, and relevance to pathophysiological conditions (e.g., fatty acid oxidation disorders and cardiac ischemia) where long-chain acylcarnitine accumulation is evident.

INTRODUCTION

Long-chain fatty acids (LCFAs) serve as an important fuel source for muscle cells, especially Type 1 “oxidative” muscle fibers and cardiomyocytes (1). Efficient fat combustion requires coincident delivery of fuel and O2 to mitochondria, with their subsequent metabolic conversions driven by energy demand. On a molecular scale, intracellular trafficking of
lipophilic metabolites through a hydrophilic environment presents an interesting problem. Fatty acid binding proteins (FABPs) have been identified in a variety of cells that solubilize, traffic and transiently sequester LCFAs (2-8). In this manner, the FABPs help regulate LCFA availability for metabolism and generation of lipid second messengers. Specifically, LCFAFs are substrates for acyl-CoA synthetases which activate LCFAFs to their CoA esters. These activated moieties flow toward mitochondrial or peroxisomal catabolism, mono-/di-/triacylglycerol synthesis, or metabolism to other derivatives such as ceramides. Acylcarnitines of various chain-lengths are formed from a carnitine molecule and their specific fatty acyl-CoA precursors through the actions of acylcarnitine transferases associated with mitochondria and peroxisomes (i.e., the carnitine shuttle) (9).

Importantly, the LCFA availability in muscle can exceed oxidative capacity, and this mismatch—as reflected in plasma or tissue concentrations of acylcarnitine markers of incomplete LCFA β-oxidation—is more apparent during exercise (10) or with insulin resistance and type 2 diabetes (11). Under the latter condition, LCFA accumulation in intramyocellular lipid (IMCL) is evident and IMCL content also correlates with insulin resistance in untrained individuals (12). Interestingly, aerobic training leads to the “athlete’s paradox” in which IMCL is increased despite excellent insulin sensitivity in muscle (13). This contrast between trained and untrained individuals is hypothesized to derive from efficient sequestration of fatty acids into triacylglycerol, where they remain relatively “inert,” thus limiting accumulation of “lipotoxic” intermediates such as ceramides or diacylglycerols (14). However, additional possibilities may be at work including training-associated changes in the expression and/or post-translational modification of sequestration proteins.

Besides being a well-known marker of incomplete β-oxidation, recent studies highlight the multifunctional role that LCFA-acylcarnitines play in the cell. These lipid moieties serve as natural zwitterions that modify membrane-associated systems or enzymes. In turn, these changes can contribute to insulin resistance, inflammation and myocyte stress responses relevant to cardiac ischemia and inborn errors of fatty acid oxidation (15-17). Despite the importance of acylcarnitines in terms of normal cellular and body-wide fuel metabolism, and their possible involvement in myocellular function (or dysfunction, when in excess), little is known about myocyte acylcarnitine trafficking and sequestration.

Myoglobin (Mb) is a single polypeptide of ~153 amino acids length. Mb is one of the most abundant proteins in muscle and cardiomyocytes (18) and its concentrations in muscle are increased by aerobic training (19). Mb has eight right-handed α-helices which are arranged in a distorted bundle orientation or a triangular prism shape structure (20-22). The arrangement of the protein is highly compact and contains a heme group (i.e., porphyrin ring with iron at its center), where the heme is held by a proximal histidine group attached directly to iron (Fe). A distal histidine group placed on the opposite side of heme is not bonded to Fe; rather, it is involved in binding small molecular ligands (23). The heme is surrounded by the neighboring non-polar amino acids in the interior of the Mb. This compact nature of Mb allows only a few small molecules to reach its interior. Molecular O₂, being non-polar, freely diffuses into the interior of the protein and binds to the heme iron. Binding of O₂ changes the Mb conformation by pulling the iron into the heme plane (i.e., in Deoxy-Mb the Fe is out of the heme plane). Hence, O₂ binding alters the orientation of the proximal histidine, resulting in a conformational change in the helix F of Oxy-Mb.

A growing body of evidence points to Mb as a protein involved in binding LCFAFs in an oxygen-dependent manner (23-25). In support, biochemical and NMR studies have demonstrated the in vitro interaction between Oxy-Mb and palmitate or oleate (26-28). Recently, our group utilized molecular dynamic structural modeling to identify the putative binding site and key amino acid residues involved in C16 fatty acid binding to Oxy-Mb, but not Deoxy-Mb (23). While it has been proposed that fatty acid binding to Mb is promoted with unsaturation and increases as a function of chain length (25), the full range of fatty acids bound by Oxy-Mb remains to be determined. Nevertheless, these observations raise the intriguing possibility that Mb helps regulate LCFA oxidative versus non-oxidative fates under dynamic physiological states (i.e., rest vs. exercise) and/or contrasting metabolic health conditions (i.e., fit insulin-sensitive vs. sedentary insulin-resistant states). In other words, Mb may be an integral part of the network of players that modulate physiologic and lipotoxic outcomes in muscle cells.

Given the LCFA and Oxy-Mb interactions, it is reasonable to consider that Oxy-Mb may bind acylcarnitines in a chain-length-specific manner as...
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well. It is further reasoned that due to the carnitine head group, the binding characteristics will differ from LCFA due to polar interactions at the interface between the Oxy-Mb hydrophobic pocket and the outside of the protein. To evaluate these hypotheses, we determined the chain-length specificity of fatty acid and fatty acylcarnitine binding to Oxy-Mb vs. Deoxy-Mb utilizing molecular dynamics (MD) simulations and isothermal titration calorimetry (ITC) experiments. Our findings support a heretofore unrecognized role of Mb in the acylcarnitine pathway and contribute to the understanding of the structural determinants, dynamics, and stability of different chain lengths and ligands with differing unsaturation.

RESULTS

AutoDock predictions

In our previous study using MD simulations, we showed the mechanistic features predicting the ligand binding site, ligand interactions, and effect(s) of oxygen binding on the heme structure that enables fatty acid binding (i.e., palmitate or oleate) to Oxy-Mb but not to Deoxy-Mb (23). In the current study, we performed molecular docking experiments involving varying chain lengths (C2 to C20) of both fatty acids and acylcarnitines to Oxy-Mb and Deoxy-Mb. Docking results for these ligands with Deoxy-Mb showed no favorable binding, consistent with previous MD studies and experimental results with LCFAs (23,27) (Fig. 1A & 1C). This lack of interaction is likely due to the effect of the dome-shaped heme and the degree of heme tilting in Deoxy-Mb, which is the result of a slight decrease in the coordination bond length between the Fe and histidine (Nε2 [epsilon nitrogen of His] and the imidazole ring) (29,30). For the Oxy-Mb simulations, our present results accord with our previous docking outcomes. Specifically, LCFAs and long-chain acylcarnitines exhibit similar binding modes, in that they bind near the porphyrin group and the hydrophobic region of the protein (Fig. 1B & 1D). This lack of interaction is likely due to the effect of the dome-shaped heme and the degree of heme tilting in Deoxy-Mb, which is the result of a slight decrease in the coordination bond length between the Fe and histidine (Nε2 [epsilon nitrogen of His] and the imidazole ring) (29,30). For the Oxy-Mb simulations, our present results accord with our previous docking outcomes. Specifically, LCFAs and long-chain acylcarnitines exhibit similar binding modes, in that they bind near the porphyrin group and the hydrophobic region of the protein (Fig. 1B & 1D). Specifically, the carboxyl head group of the fatty acid or acylcarnitine interacts with the sidechain amino group of Lys45 and Lys63, while the lipid tail region exhibits hydrophobic contacts. Estimated binding energies predicted by Autodock (see Table 1) were lower for the short- and medium-chain fatty acids and their respective acylcarnitines (i.e., C2 to C10 values < -4 kcal/mol), when compared to LCFA and long-chain acylcarnitines. Overall, we found that C2-C10 metabolite binding was not stable (discussed below).

Molecular Dynamic simulations of the various ligands in the myoglobin fatty-acid binding pocket

Our recent study, using MD simulations of Oxy-Mb complexed with palmitate and oleate, illustrated the crucial role of the fatty acid hydrophobic tail in stabilizing the entire protein-lipid complex (23). In the present study, we sought to understand the structural stability of the protein-lipid complex for different chain lengths of ligands as well as the effect of the carnitine head group in binding. Therefore, we carried out 100 ns MD simulations separately for each protein-lipid complex (12 fatty acids & 12 acylcarnitines in total). In addition, we also performed simulations using arachidonate or arachidonoylcarnitine (C20:4) to understand the effects of polyunsaturation on binding dynamics, due to the presence of four double bonds. In all cases, the starting structure has the carboxyl group of the lipid interacting with Lys45 and Lys63 of Mb enabling hydrogen bonding. Analysis of the trajectories for short- and medium-chain acylcarnitines revealed that the ligand starts to move out of the binding site within the first 10 ns simulation time and is thereafter exposed to the hydrophilic environment (Fig. 2, A-C, illustrating C4, butyroylcarnitine). Similar outcomes were observed for either fatty acid or acylcarnitine molecules ranging from C2:0 to C10:0 chain length. In other words, there was essentially no binding of either ligand with chain lengths up to C10:0 to Oxy-Mb under the conditions tested, as the tail is too short to have stable hydrophobic interactions. For both fatty acids and acylcarnitines, C12:0 is the minimum chain length required to form a stable complex with Oxy-Mb (Fig. 3A & 3F).

In our previous studies (23), stable interactions were seen between the non-polar alkyl tail of LCFAs and hydrophobic residues Leu29, Phe33, Phe43, Phe46, Val67, Val68 and Ile107 within Oxy-Mb. Conversely, the current MD trajectories on short- and medium-chain lipids reveal a failure to exhibit hydrophobic interactions during the course of the simulation run. This is due to the fact that these short tails do not interact with the deeper-sitting hydrophobic residues described above, nor are the interactions strong enough to resist thermal fluctuations and hold the ligands in the hydrophobic groove. Moreover, when a short-tail ligand emerges from the pocket, it lacks a few
terminal carbons that would coordinate the tail at the pocket entrance to facilitate the return. Eventually, the short tail region moves far enough out of the pocket, ultimately leading to the dissociation from Mb. This is in sharp contrast with the long-chain lipid moieties in that binding and retention is long-lasting. For example, palmitoylcarnitine is stabilized in the hydrophobic pocket, thereby forming hydrogen bonds; its “U” shaped tail interacts with the conserved hydrophobic residues of Mb (Fig. 2, D-F). The energetic reason for the long-chain hydrophobic tail occupying the hydrophobic groove is its highly nonpolar nature, which drives penetration away from the aqueous environment outside of the Mb molecule. In contrast, the fatty tails of short- and medium- chain lipids (C2:0 to C10:0) are not pushed into the crevice because the hydrophobic forces are roughly proportional to the exposed nonpolar area.

In MD simulations, the carboxyl head group in LCFA and long-chain acylcarnitines (i.e., C12 or longer) exhibited hydrogen-bonding interactions with Lys45 or Lys63. The results showed that these head groups switched between Lys45 and Lys63 during the 100 ns simulation, with an average dwelling time on the order of 25 ns. During the 100 ns simulations, the majority of the H-bonding is associated with Lys45, with some exceptions (Fig. 4). Overall we find that the head group of fatty acids tend to show a higher percentage of hydrogen bonding with Lys45 or Lys63 than their respective acylcarnitines (Fig. 4). In addition to this difference, the NH3+ moiety in the carnitine head group also shows interactions with residues Lys42, Lys47, Ser58, His64 and His97.

Comparative visual analysis of various ligands (C12 and above) in the hydrophobic pocket of Oxy-Mb revealed interesting features. Laurate exhibited “linear” shape conformation throughout the 100 ns simulation run, whereas lauroylcarnitine switched between a “linear” and “U” shaped conformation before attaining a final “U” shape structure (Fig. 3A). In contrast, myristate, myristoylcarnitine, palmitate, palmitoylcarnitine, oleate and oleylcarnitine exhibit the characteristic “U” shaped conformation similar to the binding conformation in the FABP proteins (Fig. 3- Panels, B-D, F-I) (31-38). Due to the presence of the single double bond at the center of the molecule in both oleate and oleylcarnitine (formation of kink in the structure due to the cis bond between C9-C10), these two ligands always display “U” shape conformation. The double-bond limits structural flexibility which allows the lipids to stabilize the interactions with the surrounding hydrophobic residues. During the MD simulations, the saturated long-chain ligands partially exhibit “linear” shape during the MD simulations before attaining the final characteristic “U” shape conformation mimicking LCFAs binding mode in FABPs (31,32). Long-chain ligands (C14:0 to C18:1) show additional hydrophobic contacts ranging from Val28, Leu29, Leu32, Phe43, Phe46, Val67, Val68, Ala71, Leu72, Leu104, Ile111 and Leu135. Similar hydrophobic interactions were observed with both arachidonate and arachidonoylcarnitine (C20:4). With respect to the latter, the presence of four cis double bonds induces categorical bends in the lipid molecule leading to it attaining an “S” shaped conformation (Fig. 3E & 3J).

We monitored the stability of the ligands by Root Mean Square Fluctuation (RMSF). We compared the RMSF trajectories between the 5 fatty acid and 5 acylcarnitine ligands that show stable binding. We found that laurate and lauroylcarnitine ligands exhibited larger movement in the binding pocket when compared to the rest of the lipids tested (Fig. 5). This may be due to presence of a relatively shorter hydrophobic tail that switches between a “linear” and “U” shaped structure. Fatty acids exhibited lesser movement in the hydrophobic core compared to acylcarnitines. Although their RMSF profiles are similar (Fig. 5), once the hydrophobic tail occupied the hydrophobic pocket, the carnitine moiety showed larger fluctuations due to the presence of the trimethyl head group, which interacts with the surrounding water molecules.

The presence of one cis double bond in oleate and oleylcarnitine and four cis-double bonds in arachidonate and arachidonoylcarnitine limits the torsional angles of these molecules. The position of the cis double bond may also influence the position or the energy cost of bending the tail while docked in the hydrophobic pocket. If the position of the double bonds in the lipids changes, the pre-disposed bend might be in the wrong place for the lipid to attain stability. For example, in the case of arachidonoylcarnitine, addition of the carnitine head group appears to strengthen the position or the energy cost of bending the tail

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molecular oxygen was further purged with nitrogen gas to attain enriched Oxy-Mb. For control preparations, Deoxy-Mb was generated by purging the Mb preparation with N₂ gas which eliminates the dissolved oxygen from the solution. Under these conditions, we found a lower binding affinity of PLM to the Deoxy-Mb enriched preparation. Specifically, the affinity of PLM for Mb decreased by approximately 50% (Kₐ values in µM: N₂ purge, 67.1 ± 3.0 versus standard condition, 32.7 ± 1.1 (data not shown)). Comparing standard conditions (i.e., sodium dithionite alone) and O₂ purge (i.e., sodium dithionite + O₂), we found no significant difference in the PLM Kₐ values. This notwithstanding, we continued to use the O₂ purge in the follow-up ITC experiments since some ligands may be more sensitive to O₂ concentration than PLM. We also tested the effect of pH and temperature on the affinity of fatty acids or acylcarnitines with Mb. Across the range of pH 6-8 or temperature 25°C to 37°C, there were no major differences in Kₐ values between PLM and Oxy-Mb (data not shown).

Importantly, the quality of the Met-Mb and Oxy-Mb samples was verified by acquiring UV-vis spectra of the samples before and after the ITC experiments. UV-vis spectra of Met-Mb showed the characteristic absorption maxima at 417 nm, and the β and α bands at 504 nm and 530 nm, respectively. In contrast, Oxy-Mb showed significant red shift of the corresponding bands (to 422 nm, 510 nm, and 560 nm, respectively, data not shown). These results are consistent with values reported in the literature for Met-Mb and Oxy-Mb-enriched solutions (39).

Having now optimized experimental parameters, we conducted ITC experiments with varied chain-lengths of ligands to determine binding to Oxy-Mb (enhanced by O₂ purge) vs Met-Mb (comprises most of commercial preparations) (Fig. 6). As a negative control, we conducted identical experiments with hen egg white lysozyme which was titrated independently with palmitate (PLM) and palmitoylcarnitine (PLC) (Fig. 6A & 6E). Lysozyme (~14 kDa) is similar molecular mass as Mb, and is known to not bind fatty acids. In accord, we found that lysozyme did not exhibit discernable binding to either of the ligands tested. Taken together, these results support the hypothesis that the interaction of LCFAs and long-chain acylcarnitines to Oxy-Mb is specific (Fig. 6B-D & 6F-H).

Notably, the variation in the solubility of different fatty acids and acylcarnitines resulted in slight changes in the peaks evolved during the injection process. This is mainly due to the effect of possible aggregation of either fatty acids or acylcarnitines during the titration process which cannot be accounted accurately. Nevertheless, appropriate background corrections were performed to eliminate heats of dilution and
potential heat changes arising due to products formed during the course of the ITC experiments (see Methods).

**ITC: Effect of chain-length of FA and AC on thermodynamic binding parameters of Oxy-Mb**

In ITC experiments, fatty acids or acylcarnitines from chain-length C6 to C10 showed no significant binding to either Met-Mb or Oxy-Mb (Table 2). In contrast, ligands with chain lengths C16:0 and C18:1 showed significant binding to Oxy-Mb but not to Met-Mb (Table 2; see Fig. 6 for representative ITC traces). A ~3-fold differential binding affinity was observed in the case of C16:0 and C18:1 fatty acids vs. acylcarnitines (Table 2). Conversely, C12:0 ligands exhibited very weak binding to Oxy-Mb with a Kd value of 509 ± 177 µM (fatty acid) and 864 ± 259 µM (acylcarnitine) due to low heat changes which may have resulted in an incomplete saturation.

Large negative enthalpic (ΔH) values for both fatty acids and acylcarnitines suggest that binding interaction to Oxy-Mb is favored (Table-2). Observing a decrease in the enthalpy and an increase in the entropy (ΔS) of fatty acids with larger tail length supports the binding of respective ligands to Oxy-Mb which is predominantly driven by hydrophobic interactions. Conversely, an opposite trend is observed in acylcarnitines, where an increase in enthalpy and decrease in entropy supports that the interactions are governed by both hydrophobic and hydrogen bonding. On an average, all the lipids studied showed a binding stoichiometry of ~1 suggesting the presence of a single, binding site in equine Oxy-Mb for either fatty acids or acylcarnitines. The binding affinity of arachidonic acid to Oxy-Mb could not be studied due to its poor solubility under the conditions used in the ITC experiment. In addition, experiments could not be performed with arachidonoylcarnitine as it was not commercially available at the time of the experiments.

**DISCUSSION**

For both fatty acids and acylcarnitines, ITC data show a consistent strengthening of binding with an increase in the length of the hydrocarbon tail, which agrees with the MD simulations and the expectation that the hydrophobic exclusion of the nonpolar tail from water and its Van der Waal interactions with the crevice are crucial factors for the complex stability.

However, there are clear distinctions between fatty acids and acylcarnitines in the enthalpic and entropic contributions to the binding energy, which can be reasonably interpreted in the context of the differences of their head group structure. Fatty acids show binding entropy increasing with the tail length. This trend is typical for hydrophobic exclusion as more water gets released from the vicinity of the tail when it enters the Mb crevice. This favorable component is partially balanced by the decreasing favorable enthalpy observed in ITC. For similar systems, the favorable binding enthalpy often comes from strong electrostatic interactions, and its waning in the case of fatty acids is likely due to decrease in the ability of the carboxyl group to engage in polar contacts with the positively charged Mb groups because the larger tail holds it deeper into the Mb crevice and restricts conformational freedom.

In contrast, in the case of acylcarnitine binding, the increase in the tail length favors the strengthening of the enthalpic contribution, while the unfavorable (negative) entropic part becomes even more pronounced. A possible structural reason for this opposite trend may be due to the head groups of the lipid moieties. Compared to the small carboxyl in fatty acids, the carnitine head group is much larger and heterogeneous in terms of atomic polarity. The carnitine adds both hydrophilic (carboxylic and trimethylammonium) and hydrophobic (two CH2 and one CH) groups to the ligand. It is reasonable to consider that this larger head group, with more charged atoms, is able to form more polar contacts with Mb. These interactions may be especially pronounced when a larger lipid tail enforces a deeper placement into the Mb crevice — the situation when the contact abilities of the fatty acid carboxyl are likely to be limited, whereas the larger carnitine head group has a farther reach and more conformational freedom. These differences may contribute to the increase in the enthalpic part of the binding energy with the tail lengthening observed in acylcarnitines. At the same time, several hydrophobic atoms present in the acylcarnitine head group might provide a possible “docking spot” for the hydrophobic tail while acyl carnitines are still in the bulk solution. In this case, wrapping of the tail to cover those carbons from water could also limit the size of the hydration shell of the nearby polar atoms in the carnitine head group. When acylcarnitines bind to Mb, the tail would unwrap, enter into the Mb crevice and thus expose non-polar carbons of the head group to water which would also allow the polar atoms of the head group to increase the hydration shell. Typically, hydration of polar atoms makes
favorable enthalpic contributions, which would agree with ITC data for acylcarnitines. Additionally, increased hydration of both polar and non-polar atoms in the “uncovered” carnitine head group is expected to cause ordering of nearby waters, a likely reason for the larger negative entropy observed with the increased tail length. Hence, affinity of fatty acids and acylcarnitines to Oxy-Mb increases with the increase in chain length, whereas the specific energetic contributions and group interactions might differ.

The results from our molecular modeling simulations demonstrate that medium- and long-chain lipids (from C12:0 to C20:4) have the ability to bind Oxy-Mb, due to the presence of the long alkyl tail which forms a “U” or “S” shaped structure occupying the hydrophobic groove of Mb. MD simulations suggest that short- and medium-chain fatty acids and acylcarnitines of C10 or below do not bind to Oxy-Mb. Calculated binding energies suggest that acylcarnitines bind relatively more favorably compared to fatty acids owing to the presence of the carnitine head group. Furthermore, the MD simulations revealed crucial Mb residues Lys45 and Lys63 anchor the charged end of lipids, while hydrophobic residues interact with the hydrophobic tail of the lipid establishing a stable conformation. Consistent results from molecular modeling and ITC studies validate the physical binding of both fatty acids and acylcarnitines to Oxy-Mb. Future experiments are warranted to study the mechanism of entry and exit, and to determine the importance of specific residues identified herein (i.e., through site-directed mutagenesis). Interestingly, recent studies indicate that Oxy-Mb interacts with mitochondria in the muscle cells, initiating a conformational change in the Oxy-Mb during the release of oxygen (40,41). These results must be taken into account in future studies of the retention of fatty acids or acylcarnitines when bound to Oxy-Mb that may simultaneously release both oxygen and lipids to the mitochondria.

The novel observation that some acylcarnitines bind to Oxy-Mb raises the intriguing possibility that Mb serves as a physiological carrier of fatty acid fuels, especially Mb-rich type I “oxidative” muscle fibers or cardiomyocytes that rely heavily on fatty acid combustion. In addition, MD simulation studies of Oxy-Mb with fatty acids and acylcarnitines suggest an important role for these metabolites in the physiological function of the myoglobin as a carrier of oxygen. Indeed, these ligands might stabilize oxygen binding as they partially occlude one of the major oxygen diffusion pathways in Mb. In support, it has been shown that such obstacles can affect the rates of oxygen release (42-44).

Long-chain acylcarnitines can accumulate in muscle: (i) during exercise, (ii) in some fatty acid oxidation disorders, (iii) in the insulin resistant state, and (iv) in cardiac ischemia. Moreover, these metabolites have been hypothesized to serve as bioactive “stress” signals when accumulation is abnormally high (10,16). It is reasonable to speculate that Oxy-Mb impacts the free intracellular concentration of these metabolites, and may be a means by which bioactive acylcarnitines are metered and trafficked in myocytes. Recently, long-chain acylcarnitines were associated with regions of tissue damage in a heart cardiac ischemia model (45). The untoward effects of acylcarnitines on mitochondrial function were ameliorated with addition of total cellular proteins but not FABP or ACBP (acyl-CoA binding protein), suggesting the presence of an unknown acylcarnitine binding protein in cardiomyocytes. Our results suggest that Oxy-Mb serves as one such protein. Under conditions of low tissue oxygenation and enrichment of tissue Deoxy-Mb (i.e., cardiac ischemia), such a model would predict that acylcarnitine binding to Mb drops, thereby raising free intracellular acylcarnitine concentrations with concomitant increases in acylcarnitine-associated cell bioactivities and stress outcomes.

EXPERIMENTAL PROCEDURES

Molecular docking

We used the identical horse Oxy-Mb structure/model coordinates that were previously used to study the palmitate (PLM) and oleate (OLE) interactions with Oxy-Mb (23). For horse (Equus caballus) Deoxy-Mb structure, we used (PDB ID: 2V1K) and due to the non-availability of horse Oxy-Mb, we transferred the oxygen molecule coordinates from sperm whale Oxy-Mb (PDB ID: 1MBO) to the Deoxy-Mb of horse (46,47). To attain equilibrium and stability, we performed MD simulations on the generated Oxy-Mb complex for a period of 10 ns before using the model for docking studies. All the ligand molecules, which include different fatty acids and acylcarnitines starting from acetic/acetyl-(C2) to arachidonic/arachidonoyl (C-20) were sketched and minimized using Marvin Sketch software (Marvin v5.7.1, 2011, ChemAxon, http://www.chemaxon.com). The difference between the fatty acid and acylcarnitine is only the addition of carnitine molecule to the head group of
the fatty acid. Molecular docking was performed using Autodock 4.2 to estimate their binding free energies and to obtain the best orientation of fatty acids and acylcarnitines in Oxy-Mb to generate the initial protein-lipid complex for MD simulations (48). A hybrid Lamarckian Genetic Algorithm (LGA) was used for all the calculations listed in the Autodock module (49). The torsional angles for the residues K45 and K63, which are involved in the binding of PLM & OLE, were kept flexible along with the torsional angles of all 24 ligand molecules (48,50). All the other residues in the Oxy-Mb were held rigid. Polar hydrogen atoms were added to the Oxy-Mb structure using AutoDock and subsequently Kollman united atom partial charges were assigned (48-50). The same input parameters used in our previous study (23) were used for the grid box settings which is set to 70 X 70 X 70 points with grid spacing of 0.375 Å. Maximum energy evaluations of 25,000,000 steps were performed with a population size of 300 while the total independent runs were fixed to 150. The clustering algorithm described in ADT/AutoDock to group the similar conformation or “clusters” based on their lowest energy conformations and their RMSD to one another is applied (48,50). The docked poses with lowest energy from each run were saved and clustered with an RMSD cut-off of 2 Å. Finally, the most energetically favorable conformation of each ligand was selected.

Molecular dynamic (MD) simulations

MD simulations were performed on all of the 24 protein-lipid complexes (12 fatty acids and 12 acylcarnitines) using NAMD (51) package developed by the Theoretical and Computational Biophysics Group at University of Illinois at Urbana-Champaign (52). All the simulations were conducted using the NPT ensemble using CHARMM36 force field parameters (53,54). Before performing the MD simulations, the protein-lipid complex was energy minimized for the AutoDock generated ligand poses. These minimizations used periodic boundary conditions, where protein-lipid complex was solvated with a TIP3 water model (55) in a rectangular 3D periodic box, of which the dimensions in every direction were chosen to be at least 10 Å larger than the protein-lipid complex. Na+ & Cl- ions were added up to equivalent of 150 mM salt concentration to each protein-lipid (Oxy-Mb/FA, Oxy-Mb/AC) complex in order to maintain the electroneutrality for all the 24 complexes. Using Langevin Dynamics, a constant pressure (1 atm) and temperature regulation (1°K to 300°K) with a collision frequency of 1.0 is maintained (56,57). For the entire MD simulation for each test, periodic boundary settings were maintained with the cutoff distance applied for non-bonded interactions taken as 12 Å, and the particle mesh Ewald (PME) method was used to treat long-range electrostatic interactions with the switching distance 1.5 Å. A three-stage protocol was employed for energy minimization of the solvated protein-lipid complex. In the first step, to avoid clashes between conflicting contacts, energy minimization was performed only on the solvent molecules keeping the protein fixed using the steepest descent in the first 3000 steps. In the second step, a conjugate gradient method for 3000 steps was employed keeping the heavy atoms of the Oxy-Mb fixed, while both the solvent and hydrogen atoms in the Oxy-Mb were allowed to relax. In the final step, all the solvent molecules and the protein atoms were allowed to relax for the subsequent 3000 steps during optimization. To achieve equilibrium, the system was subjected to steady heating until it reached 300°K at 1 atm. Throughout the MD simulations, the coordinates of each system were saved every 1 picosecond (ps). RMSD calculations were performed using the plugin RMSD trajectory tool, whereas hydrogen bonding interactions of the protein-lipid complex were analyzed using HBonds plugin in vmd 1.9.1 (52).

Isothermal titration calorimetry

All the ITC experiments were performed on iTC200 microcalorimeter (GE, Northampton, USA). Stock solutions of samples containing different chain-lengths of free fatty acids and respective acylcarnitines were prepared in 100% ethanol, and diluted to a final concentration of 50 µM in 10 mM sodium phosphate buffer, pH 7.2 containing 50 mM NaCl and 10% ethanol. Incorporation of 10% ethanol in the titration buffer increased the solubility of FAs/ACs at room temperature. At higher concentrations (>500 µM), aggregation was observed in both FAs and ACs. FAs/ACs were always loaded in the reaction cell at a concentration of 50 µM and titrated against 500 µM of Oxy/Met-Mb solution. Oxy-Mb samples (500 µM) was prepared in the same buffer by the addition of 3 mM sodium dithionite, and O2 gas was purged into the solution for 10 min. Both the protein and FA/AC samples were equilibrated to 25°C before the start of the titration. A total of 25 injections (1.6 µL each) from syringe to cell was carried out to generate the ITC curves within each
experiment. Samples were thoroughly mixed by constant stirring of 1000 rpm. Between each injection, a 10 s gap was maintained to achieve a proper baseline. Data obtained from the ITC experiments were best fit to one-set of sites binding model, available on Origin™ software (v7.0) provided by the manufacturer. Heats of dilutions and heats due to potential products formed during the course of the ITC experiments were corrected by performing appropriate blank titrations, consisting of Oxy/Met-Mb into buffer and buffer into FAs/ACs, from the actual sample titrations.

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Author Contributions: SVC and SHA conceived of acylcarnitine-Mb binding. SVC conducted all computational experiments, and interpreted MD simulation results in consultation with PLM, DVR and AA. SJ and RKG conducted ITC experiments and analyzed results with TKSK. SVC, SHA, TKSK, and SJ conceived the experimental design. SVC and SHA wrote the manuscript, with input and edits by all authors.
REFERENCES

1. Randle, P. J. (1998) Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years. *Diabetes Metab Rev* **14**, 263-283

2. Chmurzynska, A. (2006) The multigene family of fatty acid-binding proteins (FABPs): function, structure and polymorphism. *J Appl Genet* **47**, 39-48

3. Dutta-Roy, A. K. (2000) Cellular uptake of long-chain fatty acids: role of membrane-associated fatty-acid-binding/transport proteins. *Cell Mol Life Sci* **57**, 1360-1372

4. Glatz, J. F., and van der Vusse, G. J. (1990) Nomenclature of fatty acid-binding proteins. *Mol Cell Biochem* **98**, 231-235

5. Glatz, J. F., and van der Vusse, G. J. (1990) Cellular fatty acid-binding proteins: current concepts and future directions. *Mol Cell Biochem* **98**, 237-251

6. Ockner, R. K. (1990) Historic overview of studies on fatty acid-binding proteins. *Mol Cell Biochem* **98**, 3-9

7. Kaikaus, R. M., Bass, N. M., and Ockner, R. K. (1990) Functions of fatty acid binding proteins. *Experientia* **46**, 617-630

8. Spener, F., Unterberg, C., Borchers, T., and Grosse, R. (1990) Characteristics of fatty acid-binding proteins and their relation to mammary-derived growth inhibitor. *Mol Cell Biochem* **98**, 57-68

9. Sharma, S., and Black, S. M. (2009) Carnitine Homeostasis, Mitochondrial Function, and Cardiovascular Disease. *Drug Discov Today Dis Mech* **6**, e31-e39

10. Adams, S. H., Hoppel, C. L., Lok, K. H., Zhao, L., Wong, S. W., Minkler, P. E., Hwang, D. H., Newman, J. W., and Garvey, W. T. (2002) Plasma acylcarnitine profiles suggest incomplete long-chain fatty acid beta-oxidation and altered tricarboxylic acid cycle activity in type 2 diabetic African-American women. *The Journal of nutrition* **139**, 1073-1081

11. Koves, T. R., Ussher, J. R., Noland, R. C., Slentz, D., Mosedale, M., Ilkayeva, O., Bain, J., Stevens, R., Dyck, J. R., Newgard, C. B., Lopaschuk, G. D., and Muoio, D. M. (2008) Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab* **7**, 45-56

12. McGarry, J. D. (2002) Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. *Diabetes* **51**, 7-18

13. Dube, J. J., Amati, F., Stefanovic-Racic, M., Toledo, F. G., Sauers, S. E., and Goodpaster, B. H. (2008) Exercise-induced alterations in intramyocellular lipids and insulin resistance: the athlete's paradox revisited. *American journal of physiology. Endocrinology and metabolism* **294**, E882-888

14. Aon, M. A., Bhatt, N., and Cortassa, S. C. (2014) Mitochondrial and cellular mechanisms for managing lipid excess. *Front Physiol* **5**, 282

15. Rutkowsky, J. M., Knotts, T. A., Ono-Moore, K. D., McCain, C. S., Huang, S., Schneider, D., Singh, S., Adams, S. H., and Hwang, D. H. (2014) Acylcarnitines activate proinflammatory signaling pathways. *American journal of physiology. Endocrinology and metabolism* **306**, E1378-1387

16. Aguer, C., McCain, C. S., Knotts, T. A., Thrush, A. B., Ono-Moore, K., McPherson, R., Dent, R., Hwang, D. H., Adams, S. H., and Harper, M. E. (2014) Acylcarnitines: potential implications for skeletal muscle insulin resistance. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*

17. McCain, C. S., Knotts, T. A., and Adams, S. H. (2015) Acylcarnitines--old actors auditioning for new roles in metabolic physiology. *Nat Rev Endocrinol* **11**, 617-625

18. Nelson, D. (2000) Lehninger Principles of Biochemistry. *New York: Worth Publishers 3rd edition*

19. Terjung, R. L. (1995) Muscle adaptations to aerobic training. *Sports Science Exchange* **8**, 1-4
Lipids binding to Myoglobin

20. Kendrew, J. C., Bodo, G., Dintzis, H. M., Parrish, R. G., Wyckoff, H., and Phillips, D. C. (1958) A three-dimensional model of the myoglobin molecule obtained by x-ray analysis. *Nature* 181, 662-666

21. Vinogradov, S. N., Walz, D. A., Pohajdak, B., Moens, L., Kapp, O. H., Suzuki, T., and Trotman, C. N. (1993) Adventitious variability? The amino acid sequences of nonvertebrate globins. *Comp Biochem Physiol B 106*, 1-26

22. Suzuki, T., and Imai, K. (1998) Evolution of myoglobin. *Cell Mol Life Sci* 54, 979-1004

23. Chintapalli, S. V., Bhardwaj, G., Patel, R., Shah, N., Patterson, R. L., van Rossum, D. B., Anishkin, A., and Adams, S. H. (2015) Molecular dynamic simulations reveal the structural determinants of Fatty Acid binding to oxy-myoglobin. *PLoS One* 10, e0128496

24. Gloster, J., and Harris, P. (1977) Fatty acid binding to cytoplasmic proteins of myocardium and red and white skeletal muscle in the rat. A possible new role for myoglobin. *Biochemical and biophysical research communications* 74, 506-513

25. Gotz, F. M., Hertel, M., and Groschel-Stewart, U. (1994) Fatty acid binding of myoglobin depends on its oxygenation. *Biological chemistry Hoppe-Seyler* 375, 387-392

26. Sriram, R., Kreutzer, U., Shih, L., and Jue, T. (2008) Interaction of fatty acid with myoglobin. *FEBS letters* 582, 3643-3649

27. Shih, L., Chung, Y., Sriram, R., and Jue, T. (2014) Palmitate interaction with physiological states of myoglobin. *Biochimica et biophysica acta* 1840, 656-666

28. Shih, L., Chung, Y., Sriram, R., and Jue, T. (2015) Interaction of myoglobin with oleic acid. *Chem Phys Lipids* 191, 115-122

29. Takano, T. (1977) Structure of myoglobin refined at 2-0 A resolution. I. Crystallographic refinement of metmyoglobin from sperm whale. *Journal of molecular biology* 110, 537-568

30. Takano, T. (1977) Structure of myoglobin refined at 2-0 A resolution. II. Structure of deoxymyoglobin from sperm whale. *Journal of molecular biology* 110, 569-584

31. He, Y., Yang, X., Wang, H., Estefan, R., Francis, F., Kodukula, S., Storch, J., and Stark, R. E. (2007) Solution-state molecular structure of apo and oleate-liganded liver fatty acid-binding protein. *Biochemistry* 46, 12543-12556

32. Thompson, J., Winter, N., Terwey, D., Bratt, J., and Banaszak, L. (1997) The crystal structure of the liver fatty acid-binding protein. A complex with two bound oleates. *J Biol Chem* 272, 7140-7150

33. Lassen, D., Lucke, C., Kveder, M., Meszarzadeh, A., Schmidt, J. M., Specht, B., Lezius, A., Spener, F., and Ruterjans, H. (1995) Three-dimensional structure of bovine heart fatty-acid-binding protein with bound palmitic acid, determined by multidimensional NMR spectroscopy. *Eur J Biochem* 230, 266-280

34. Ory, J. J., and Banaszak, L. J. (1999) Studies of the ligand binding reaction of adipocyte lipid binding protein using the fluorescent probe 1, 8-anilinonaphthalene-8-sulfonate. *Biophys J* 77, 1107-1116

35. Steele, R. A., Emmert, D. A., Kao, J., Hodsdon, M. E., Frieden, C., and Cistola, D. P. (1998) The three-dimensional structure of a helix-less variant of intestinal fatty acid-binding protein. *Protein Sci* 7, 1332-1339

36. Hodsdon, M. E., Ponder, J. W., and Cistola, D. P. (1996) The NMR solution structure of intestinal fatty acid-binding protein complexed with palmitate: application of a novel distance geometry algorithm. *Journal of molecular biology* 264, 585-602

37. Zanotti, G., Spac, G., Spadon, P., Veerkamp, J. H., and Sacchettini, J. C. (1992) Three-dimensional structure of recombinant human muscle fatty acid-binding protein. *J Biol Chem* 267, 18541-18550
38. Sacchettini, J. C., Gordon, J. I., and Banaszak, L. J. (1989) Refined apoprotein structure of rat intestinal fatty acid binding protein produced in Escherichia coli. *Proc Natl Acad Sci U S A* **86**, 7736-7740

39. Eisert, W. G., Degenkolb, E. O., Noe, L. J., and Rentzepis, P. M. (1979) Kinetics of carboxymyoglobin and oxymyoglobin studied by picosecond spectroscopy. *Biophys J* **25**, 455-464

40. Yamada, T., Furuichi, Y., Takakura, H., Hashimoto, T., Hanai, Y., Jue, T., and Masuda, K. (2013) Interaction between myoglobin and mitochondria in rat skeletal muscle. *Journal of applied physiology* **114**, 490-497

41. Postnikova, G. B., Tselikova, S. V., and Shekhovtsova, E. A. (2009) Myoglobin and mitochondria: oxymyoglobin interacts with mitochondrial membrane during deoxygenation. *Biochemistry (Mosc)* **74**, 1191-1202

42. Birukou, I., Schweers, R. L., and Olson, J. S. (2010) Distal histidine stabilizes bound O2 and acts as a gate for ligand entry in both subunits of adult human hemoglobin. *J Biol Chem* **285**, 8840-8854

43. Perutz, M. F., and Mathews, F. S. (1966) An x-ray study of azide methaemoglobin. *Journal of molecular biology* **21**, 199-202

44. Scott, E. E., Gibson, Q. H., and Olson, J. S. (2001) Mapping the pathways for O2 entry into and exit from myoglobin. *J Biol Chem* **276**, 5177-5188

45. Liepinsh, E., Makreka-Kuka, M., Volska, K., Kuka, J., Makarova, E., Antone, U., Sevostjanovs, E., Vilskersts, R., Strods, A., Tars, K., and Dambrova, M. (2016) Long-chain acylcarnitines determine ischaemia/reperfusion-induced damage in heart mitochondria. *Biochem J* **473**, 1191-1202

46. Phillips, S. E. (1980) Structure and refinement of oxymyoglobin at 1.6 A resolution. *Journal of molecular biology* **142**, 531-554

47. Hersleth, H. P., Uchida, T., Rohr, A. K., Teschner, T., Schunemann, V., Kitagawa, T., Trautwein, A. X., Gorbitz, C. H., and Andersson, K. K. (2007) Crystallographic and spectroscopic studies of peroxide-derived myoglobin compound II and occurrence of protonated FeIV O. *J Biol Chem* **282**, 23372-23386

48. Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., and Olson, A. J. (2009) AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *Journal of computational chemistry* **30**, 2785-2791

49. Garrett M. Morris, D. S. G., Robert S. Halliday, Ruth Huey, William E. Hart, Richard K. Belew, Arthur J. Olson. (1998) Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *Journal of computational chemistry* **19**, 1639-1662

50. Goodsell, D. S., Morris, G. M., and Olson, A. J. (1996) Automated docking of flexible ligands: applications of AutoDock. *Journal of molecular recognition : JMR* **9**, 1-5

51. Phillips, J. C., Braun, R., Wang, W., Gumbart, J., Tajkhorshid, E., Villa, E., Chipot, C., Skeel, R. D., Kale, L., and Schulten, K. (2005) Scalable molecular dynamics with NAMD. *Journal of computational chemistry* **26**, 1781-1802

52. Humphrey, W., Dalke, A., and Schulten, K. (1996) VMD: visual molecular dynamics. *Journal of molecular graphics* **14**, 33-38, 27-38

53. Klauda, J. B., Venable, R. M., Freites, J. A., O’Connor, J. W., Tobias, D. J., Mondragon-Ramirez, C., Vorobyov, I., Mackerell, A. D., Jr., and Pastor, R. W. (2010) Update of the CHARMM all-atom additive force field for lipids: validation on six lipid types. *The journal of physical chemistry. B* **114**, 7830-7843

54. Mackerell, A. D., Bashford, D. Jr, M., Dunbrack, R. L. Jr., Evanseck, J.D., Field, M.J., Fischer, S., Gao, J., Guo, H., Ha, S., Joseph-McCarthy, D., Kuchnir, L., Kuczera, K., Lau, F.T.K., Mattos, C., Michnick, S., Ngo, T., Nguyen, D.T., Prodhom, B., Reiher, W.E., Roux,III, B., Schlenkrich, M., Smith, J. C.,Stote, R., Straub, J., Watanabe, M., Wiórkiewicz-Kuczerka, J., Yin, D., and Karplus,
Lipids binding to Myoglobin

M. . (1998) All-Atom Empirical Potential for Molecular Modeling and Dynamics Studies of Proteins. The Journal of Physical Chemistry B 102, 3568-3616

55. William L. Jorgensen, J. C., Jeffry D. Madura, Roger W. Impey and Michael L. Klein. (1983) Comparison of simple potential functions for simulating liquid water. The Journal of Chemical Physics 79, 926-935

56. Glenn J. Martyna, D. J. T. a. M. L. K. (1994) Constant pressure molecular dynamics algorithms. The Journal of Chemical Physics 101, 4177-4189

57. Scott E. Feller, Y. Z., Richard W. Pastor and Bernard R. Brooks. (1995) Constant pressure molecular dynamics simulation: The Langevin piston method. The Journal of Chemical Physics 103, 4613-4621

FOOTNOTES
The abbreviations used are molecular dynamics (MD), long-chain fatty acids (LCFAs), fatty acids (FAs), acylcarnitines (ACs), isothermal titration calorimetry (ITC), oxymyoglobin (Oxy-Mb), metmyoglobin (Met-Mb), deoxymyoglobin (Deoxy-Mb), fatty acid binding protein (FABP)

FIGURE LEGENDS

FIGURE 1. Cluster analysis from AutoDock, displaying docking conformations of horse- (A) Deoxy-Mb with PLM, (B) Oxy-Mb with PLM, (C) Deoxy-Mb with PLC, and (D) Oxy-Mb with PLC. The best 20 models for PLM and PLC are displayed as teal-blue lines and each representative set representative model is displayed as a stick. Similar patterns were observed for all the different chain lengths starting from C12 and up to C20, where the ligands for Deoxy-Mb are docked close to the lysine residues (K56 & K50) and the ligands for Oxy-Mb were placed near the porphyrin ring and the hydrophobic core close to the residues (K45 and K63).

FIGURE 2. Snapshots of horse Oxy-Mb with butyroylcarnitine (A-C) and palmitoylcarnitine (D-F) over time during MD simulations. (A) 0 ns (B) 3 ns and (C) 6 ns are the time intervals for butyroylcarnitine and (D) 0 ns (E) 50 ns and (F) 100 ns are the time intervals for palmitoylcarnitine. The protein backbone is represented as ribbon shape, whereas butyroylcarnitine (orange), palmitoylcarnitine (purple), Lys45, Lys63 and heme are displayed as sticks. Oxygen molecules are displayed as red ball shapes adjacent to the heme moiety. Butyroylcarnitine (circled) leaves the binding pocket rapidly, whereas palmitoylcarnitine remains stable in the hydrophobic pocket. Water molecules and ions are excluded for clarity.

FIGURE 3. Structure of horse Oxy-Mb interacting with fatty acids; (A) laurate (B) myristate (C) palmitate (D) oleate (E) arachidonate and acylcarnitines; (F) lauroylcarn (G) myristoylcarn (H) palmitoylcarn (I) oleoylcarn (J) arachidonoylcarn. Snapshots of only the fatty acid binding pocket is shown, with various ligands occupying the hydrophobic core near the porphyrin ring. The protein backbone is represented as ribbon (colored pink), heme as sticks (colored grey) and ligands as sticks (colored cyan). All the ligands appear the characteristic “U” shaped structure which warps around the oxygen molecule (colored red) with the exception of laurate (A) having “linear” and (E) arachidonate and (J) arachidonoylcarn attaining “S” shaped structure.

FIGURE 4. Histograms of percent of hydrogen-bond occupancies for interactions between different ligands with basic residues Lys 45 and Lys 63 of horse Oxy-Mb, across 100 ns MD simulations.
Figure 5. The averaged RMSF (Root Mean Square Fluctuation) of different fatty acids and acylcarnitines that stably bind Oxy-Mb, as a function of time bound to Oxy-Mb, showing the deviations by residue. Only heavy atoms (carbon, oxygen & nitrogen) are taken into consideration while calculating RMSF (hydrogens were excluded). Total number of atoms in each ligand molecule; laurate (14), myristate (16), palmitate (18), oleate (20), arachidonate (22), lauroylcarnitine (24), myristoylcarnitine (26), palmitoylcarnitine (28), oleoylcarnitine (30) and arachidonoylcarnitine (32).

Figure 6. Isothermograms representing the binding of lysozyme, Met-Mb and Oxy-Mb with different chain-lengths of fatty acids and acylcarnitines. Within each metabolite isothermogram, the upper panel depicts the raw data of titration of reactants with time in minutes on the x-axis and the energy released/absorbed per second on the y-axis. The lower panel is the integrated data with molar ratio on the x-axis and energy released/absorbed per injection on the y-axis. The solid line in the bottom panels represents the best-fit of the experimental data, using a one-set of sites binding model from MicroCal Origin™.

Table 1: Estimated binding energy (BE) results for different chain lengths of both fatty acids (FAs) and acylcarnitines (ACs) with Oxy-myoglobin (Oxy-Mb) from AutoDock.

| Length | Name      | Estimated BE’s (kcal/mol) |
|--------|-----------|---------------------------|
|        | FAs       | ACs                       |
| C2:0   | acetic    | acetyl                    | -3.54 | -3.77 |
| C4:0   | butyric   | butyroyl                  | -3.61 | -3.81 |
| C6:0   | caproic   | caproyl                   | -3.72 | -3.85 |
| C8:0   | caprylic  | capryloyl                 | -3.84 | -3.93 |
| C10:0  | capric    | caprioyl                  | -3.90 | -4.01 |
| C12:0  | lauric    | lauroyl                   | -4.52 | -5.03 |
| C14:0  | myristic  | myristoyl                 | -4.75 | -5.97 |
| C16:0  | palmitic  | palmitoyl                 | -5.99 | -6.16 |
| C18:1  | oleic     | oleoyl                    | -6.09 | -6.43 |
| C20:4  | arachidonic | arachidonoyl             | -6.81 | -7.33 |
Table 2:

| Samples Chain length | met-Mb Kd (app) µM | oxy-Mb Kd (app) µM | $\Delta H$ (kcal·mol$^{-1}$) | $\Delta S$ (cal·mol$^{-1}$·K$^{-1}$) | No. of binding sites |
|----------------------|-------------------|-------------------|-----------------|-----------------|-------------------|
| C6:0                 | NSB               | NSB               | NSB             | NSB             | NSB               |
| C8:0                 | NSB               | NSB               | NSB             | NSB             | NSB               |
| C10:0                | NSB               | NSB               | NSB             | NSB             | NSB               |
| C12:0                | NSB               | 509 ± 177         | -8.01           | -12.1           | 1.6 ± 0.4*        |
| C16:0                | NSB               | 29.1 ± 4.1        | -6.81           | -2.14           | 0.76 ± 0.12       |
| C18:1                | NSB               | 6.17 ± 1.6        | -4.33           | 9.32            | 0.83 ± 0.42       |
| C6:0                 | NSB               | NSB               | NSB             | NSB             | NSB               |
| C8:0                 | NSB               | NSB               | NSB             | NSB             | NSB               |
| C10:0                | NSB               | NSB               | NSB             | NSB             | NSB               |
| C12:0                | NSB               | 864 ± 259         | -4.2            | -0.07           | 1.5 ± 0.33*       |
| C16:0                | NSB               | 9.76 ± 2.6        | -7.7            | -2.9            | 0.98 ± 0.11       |
| C18:1                | NSB               | 1.98 ± 0.6        | -1.1            | -10.8           | 0.94 ± 0.18       |

NSB – No Specific Binding detected

* Due to incomplete saturation, the n value is greater than 1
FIGURE 2.

(A)  (B)  (C)

(D)  (E)  (F)
FIGURE 3.

(A) laurate  (B) myristate  (C) palmitate  (D) oleate  (E) arachidonate

(F) lauroylcarn  (G) myristoylcarn  (H) palmitoylcarn  (I) oleylcarn  (J) arachidonoylcarn
FIGURE 4.

% of hydrogen-bond occupancies

- Lys45
- Lys63

- Laurate 0.7
- Myristate 0.6
- Myristoyl carnitine 4.6
- Palmitate 4.7
- Palmitoyl carnitine 0.9
- Oleate 12.6
- Oleoyl carnitine 19.5
- Arachidonate 17.7
- Arachidonoyl carnitine 17.4
FIGURE 5.

**Fatty Acids**
- Laurate (14)
- Myristate (16)
- Palmitate (18)
- Oleate (20)
- Arachidonate (22)

**Acylcarnitines**
- Lauroylcarn (24)
- Myristoylcarn (26)
- Palmitoylcarn (28)
- Oleoylcarn (30)
- Arachinodoylcarn (32)
FIGURE 6.

**Fatty acids**

(A) C16:0  
(B) C12:0  
(C) C16:0  
(D) C18:1

Lysozyme  Met-Mb  Oxy-Mb  Met-Mb  Oxy-Mb  Met-Mb  Oxy-Mb

**Acylcarnitines**

(E) C16:0  
(F) C12:0  
(G) C16:0  
(H) C18:1

Lysozyme  Met-Mb  Oxy-Mb  Met-Mb  Oxy-Mb  Met-Mb  Oxy-Mb

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