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Interaction of myoglobin with oleic acid

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ABSTRACT

Previous studies have shown that palmitate (PA) can interact with myoglobin (Mb). The present study has investigated the interaction of the more soluble unsaturated fatty acid, oleic acid (OA). Indeed, ^{1}H NMR measurements of the Mb signal during OA titration also show signal changes consistent with specific and non-specific binding. At OA:Mb ratio < 4:1, OA perturbs selectively the 8-heme methyl signal, consistent with a local and specific fatty acid-protein interaction. As OA:Mb ratio increases from 4:1 to 40:1, all hyperfine shifted MbCN signals decrease, consistent with a non-selective, global perturbation of the protein. The pH titration analysis indicates that the observed OA methylene signal in the presence of Mb reflects a non-specific interaction and does not originate from a shift in the lamella–micelle equilibrium. Given the OA interaction with Mb, a fatty acid flux model suggests that Mb can play a fatty acid transport role under certain physiological conditions.

1. Introduction

Even though biochemistry canon asserts an O_2 storage and a facilitated O_2 transport role for Mb, many questions still abound about its cellular function (Gros et al., 2010; Wittenberg and Wittenberg, 1989, 2003). Experiments have certainly detected Mb supplying O_2 in plants and in mammalian tissue, and *in vivo* NMR experiments have observed Mb releasing its O_2 store to maintain oxidative metabolism during apnea and at the initiation of skeletal muscle contraction (Chung et al., 2005; Ponganis et al., 2002). Yet, under anoxia the O_2 store of Mb in mammalian heart can only prolong normal respiration for only a few seconds (Chung and Jue, 1996). CO inactivation of Mb function does not impair cardiac respiration, metabolism or contraction (Chung et al., 2006; Glabe et al., 1998). Indeed, a mouse without Mb exhibits no striking deficits in its oxygen consumption rate, contractile function, bioenergetics, and metabolism (Garry et al., 1998; Godecke et al., 1999). Despite compensating physiological mechanisms, the knock-out (KO) mice have led to controversial suppositions about a nitric oxide (NO) bioscavenging and NO reduction role for Mb (Flogel et al., 2001; Kreutzer and Jue, 2004, 2006; Rassaf et al., 2007).

In the Mb KO myocardium, metabolism switches its substrate preference from fatty acid (FA) to glucose. FA to glucose utilization ratio drops from 3/1 to 0.7/1 (Flogel et al., 2005). Many researchers have simply ascribed the metabolic switch to the missing contribution of Mb in facilitating O_2 transport, since oxidative fatty acid metabolism requires O_2. However, several studies have now shown that Mb in the cell appears to diffuse too slowly to compete effectively with free O_2 (Lin et al., 2007a,b; Papadopoulos et al., 1995, 2001). If Mb does not have a predominant storage or facilitated diffusion O_2 under normal physiological conditions, the reduced fatty acid metabolism could indicate a missing Mb mediated fatty acid transport, as some early Mb studies have implicated (Gloster, 1977; Gloster and Harris, 1977; Gotz et al., 1994).

Indeed, recent ^{1}H NMR studies have found evidence for palmitate (PA) binding specifically and non-specifically with Fe (III) MbCN (Sriram et al., 2008). Moreover, PA interacts differentially with physiological states of Mb. PA interacts with MbCO but does not interact with deoxy Mb. The results suggest that Mb could serve as a fatty acid transporter and use a convenient loading-unloading mechanism that follows the O_2 gradient from the sarcolemma to the mitochondria. In fact, a fatty acid transport model indicates that Mb can compete effectively with fatty acid binding protein (FABP) above a fatty acid concentration threshold (Shih et al., 2014).

To corroborate and characterize the fatty acid interaction with Mb, we have conducted a set of experiments with oleic acid (OA), an 18 carbon (C18) unsaturated fatty acid. Because OA has a higher solubility than PA, it permits the use of a wider range of fatty acid concentrations to titrate into Mb. As with PA, OA interacts specifically and non-specifically with MbCN to perturb selectively the 8-heme methyl signal (La Mar et al., 1983). At high OA:Mb, OA perturbs the overall Mb structure.

Abbreviations: OA, oleic acid; FABP, fatty acid binding protein; Mb, myoglobin; MbCN, cyanometmyoglobin.

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The experimental results confirm that Mb can interact specifically and non-specifically with OA without forming a detectable amount of hemichrome. Moreover, the Mb interaction with OA does not appear to arise from any alteration in the lamella–micelle equilibrium. Given the specific interaction of OA, a model of intracellular fatty acid transport indicates that Mb can compete with fatty acid binding protein (FABP) to transport both saturated and unsaturated fatty acid in the cell (Glatz and van der Vusse, 1989; Lin et al., 2007a,b; Shih et al., 2014; Sriram et al., 2008). Mb may then have a role in regulating fatty acid metabolism and presents a unique model to interrogate protein-fatty acid interaction.

2. Experimental

2.1. Protein preparation

Mb was prepared from lyophilized horse heart protein (Sigma Chemical Inc., St. Louis, MO). (Kreutzer et al., 1993). All the samples were prepared in 30 mM Tris buffer with 1 mM EDTA at pH 7.4. The pH was measured at 35 °C using a calomel electrode (Orion 7110BN Micro Calomel pH, Thermo Electron Corporation). Five times excess KCN was added to the metmyoglobin in Tris to produce MbCN, and the pH was adjusted to 7.4.

A UVIKON (941KONTRON Instruments) spectrophotometer measured the MbCN absorbance from 300 to 650 nm. Specifically, extinction coefficient of the 542 nm absorbance ($\varepsilon 542 = 11.3\ mM^{-1}\ cm^{-1}$) provided the basis to determine the MbCN concentration.

2.2. Fatty acid-Mb preparation

OA (Sigma Chemical Inc., St. Louis, MO) and $^{13}C_1$ OA (Cambridge Isotope, Tewksbury, MA) were dissolved in 30 mM Tris buffer with 1 mM EDTA at pH 8.5 at 65 °C. Stock solutions of 10 mM and 100 mM were prepared and kept in a heating block (Thermolyne 17600 Dri-Bath) at 65 °C. An aliquot of 10 mM or 100 mM OA in Tris buffer at 65 °C was added to 600 μl of 0.2–0.8 mM myoglobin at 35 °C to yield a final solution with Mb:OA ratios from 1:0.1 to 1:60. In these experiments, the methodological approach appears more effective and efficient than the alternative procedure that requires first dissolving a fatty acid solution in the NMR tube, evaporating the organic solvent, and then adding stoichiometric amount of protein to create the final fatty acid: protein mixture (Cistola et al., 1987).

The time between OA addition and the start of the NMR measurement was approximately 5 min. The pH was measured at 35 °C using a calomel electrode. All NMR experiments were then conducted at 35 °C.

2.3. NMR

A Bruker Avance 600 MHz spectrometer recorded the ^1H NMR signals. The 5 mm probe ^1H 90° pulse, calibrated against the HOD signal from a 0.15 M NaCl solution, was 9 μs. Watergate pulse sequence was used to suppress the water signal. Sodium-3-(trimethylsilyl) propionate 2,2,3,3 d4 (TSP) served as the internal chemical shift and concentration reference. All samples contained 10% D_2O to enable the deuterium lock during signal acquisition. All measurements were carried out at 35 °C. A typical spectrum required 1024 scans and used the following signal acquisition parameters: 36 kHz spectral width, 8k data points, and 124 ms recycle time.

The ^{13}C signals collected at 151 MHz used the following acquisition parameters: 8.25 μs 90 pulse, a 33 kHz spectral window, and 16 K data point and 1.25 s recycle time. A GARP pulse sequence decoupled the ^1H signals, and $^{13}C_2$ acetate provided an internal chemical shift reference at 24.2 ppm. Zero-filling the free induction decay (FID) and apodizing with an exponential window function improved the spectra. A spline fit then smoothed the baseline.

2.4. pK determination

NMR determination of pK used the following equation that relates the observed chemical shift to pH and pK_a:

$$\delta_{observed} = \delta_p + \frac{\delta_d - \delta_p}{1 + 10^{[pK_a - pH]}}$$

$\delta_{observed}$ = observed chemical shift at a given pH, δ_p = chemical shift of the protonated form, δ_d = chemical shift of the deprotonated form.

2.5. Fatty acid binding affinity

The curve fit used the equation $[OA]_{bound} = (B_{1max}[OA])/(K_{d1} + [OA])$ to determine a one site specific binding of OA to Mb based on the signal intensity loss of the 8 heme methyl where OA:Mb < 2:1.

[OA] = oleate; K_d = dissociation constant; and B_{max} = maximum capacity for oleate binding. The analysis assumes that the maximum signal loss of the 8-heme methyl corresponds to B_{1max} or the fully OA bound state.

Modeling a 2-site binding (a specific and a non-specific binding site) used the following equation:

$$[OA]_{bound} = \frac{B_{1max}[OA]}{K_{d1} + [OA]} + \frac{B_{2max}[OA]}{K_{d2}} = \frac{B_{1max}[OA]}{K_{d1} + [OA]} + Ns[OA]$$

At high OA concentration, the analysis assumes $Ns = B_{2max}/K_{d2}$ in the region where $K_{d2} \ll [OA]$ (Mendel and Mendel, 1985).

2.6. Intracellular fatty acid transport

The intracellular fatty acid flux has contributions from free OA and protein mediated OA diffusion as expressed in the following equation, which assumes zero unbound OA at the mitochondrial surface:

$$J = D_{OA}OA + D_X C_X \frac{OA}{K_D^X + OA}$$

J = the overall fatty acid flux, OA = oleic acid; C_X = cellular concentration of Mb ($[Mb]_{cell}$) or FABP ($[FABP]_{cell}$); D_{OA} = diffusion coefficient of free oleic acid, D_X = diffusion coefficient of Mb (D_{Mb}) or FABP (D_{FABP}) in the cell, K_D^X = *in vitro* OA dissociation constant of Mb or FABP (Lin et al., 2007a,b; Luxon and Weisiger, 1993; Richieri et al., 1994a; Sriram et al., 2008). Because the reported diffusion coefficients of fatty acid in the cell vary widely from $3.5 \times 10^{-9}\ cm^2\ s^{-1}$ to $4.6 \times 10^{-6}\ cm^2\ s^{-1}$, the model has used the highest value to set an upper bound for unbound OA contribution (Luxon and Weisiger, 1993; Vork et al., 1993). The model also assumes an identical *in vitro* and *in vivo* K_D^X. Table

2.7. Statistical analysis

Statistical analysis used the Sigma Plot/Sigma Stat program (Systat Software, Inc., Point Richmond, CA) and expressed the data as mean value ± standard error (SE). Nonlinear regression analysis of the average data points determined the dissociation constant using Marquardt-Levenberg algorithm. Statistical significance was determined by two-tailed student's *t*-test, $P < 0.05$.

3. Results

The addition of OA to MbCN produces a selective signal intensity loss of the 8 heme methyl resonance (inset figure of heme). Fig. 1