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Bridging the gap between chemistry, physiology, and evolution: Quantifying the functionality of sperm whale myoglobin mutants

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ABSTRACT

This work merges a large set of previously reported thermochemical data for myoglobin (Mb) mutants with a physiological model of O_2-transport and -storage. The model allows a quantification of the functional proficiency of myoglobin (Mb) mutants under various physiological conditions, *i.e.* O_2-consumption rate resembling workload, O_2 partial pressure resembling hypoxic stress, muscle cell size, and Mb concentration, resembling different organism-specific and compensatory variables. We find that O_2-storage and -transport are distinct functions that rank mutants and wild type differently depending on O_2 partial pressure. Specifically, the wild type is near-optimal for storage at all conditions, but for transport only at severely hypoxic conditions. At normoxic conditions, low-affinity mutants are in fact better O_2-transporters because they still have empty sites for O_2, giving rise to a larger [MbO_2] gradient (more varying saturation curve). The distributions of functionality reveal that many mutants are near-neutral with respect to function, whereas only a few are strongly affected, and the variation in functionality increases dramatically at lower O_2 pressure. These results together show that conserved residues in wild type (WT) Mb were fixated under a selection pressure of low P_{O2}.

1. Introduction

Due to its central position in protein science, myoglobin (Mb) is often called the 'hydrogen atom of biology' (Frauenfelder et al., 2003). As the first protein whose three-dimensional structure was revealed at atomic resolution (Kendrew et al., 1958), it has been the subject of extensive experimental and theoretical research. Mb is a monomeric protein of approximately 16 kDa which is generally abundant in muscles (both cardiac and skeletal) of vertebrates and in the body wall of invertebrates (Suzuki and Imai, 1998). Mb increases the availability of oxygen in muscle cells by taking up the oxygen released by hemoglobin in blood, thereby providing O_2 during muscle contraction, when blood flow through capillaries is restricted. A cornerstone of muscle metabolism is thus the ability of Mb to enhance the supply of O_2 to the mitochondria (Wittenberg, 1970).

Direct storage of O_2 has been considered the main function of Mb, based on the observation of elevated concentrations of Mb in diving mammals (Guyton et al., 1995; Ponganis et al., 2002) and increased expression of Mb at higher altitudes (Gimenez et al., 1977; Terrados et al., 1990). In contrast, it has been a matter of controversy whether Mb can contribute significantly to active O_2 transport within the cell: Despite the smaller diffusion coefficient of Mb compared to free O_2, the high concentration of Mb in working heart and muscle cells, more than thirty-fold than that of free O_2, confers an advantage in transporting O_2 from the sarcolemma to the mitochondria (Wittenberg and Wittenberg, 2003). *In vitro* studies have indeed confirmed that O_2 diffuses faster in Mb solution than in Mb-free solution (Wittenberg and Wittenberg, 1989), and Mb exhibits sufficient mobility and O_2-carrying ability to compete effectively with free O_2. *In vivo*, however, the role of Mb in O_2 diffusion has remained obscure for decades (Gros et al., 2010).

From a theoretical perspective, models have elaborated on the classical work by Krogh (1919) to clarify the problem (Groebe, 1995). Since these models were based on variable quantities such as concentrations and diffusion coefficients in muscle tissues, acceptance of Mb-facilitated diffusion has languished in absence of definitive experimental confirmation (Lin et al., 2007a).

Recently, pulsed-field gradient NMR and Flourescence Recovery After Photobleaching (FRAP) methods enabled precise measurement of the endogenous diffusion coefficient of Mb in myocardial and skeletal muscle cells (Papadopoulos et al., 2001; Lin et al., 2007a,b). Following these results, it was then argued that Mb probably has no significant contribution to O_2 transport under normoxic conditions, while its importance increases as the oxygen pressure declines and the cell experiences hypoxia.

Mb binds O_2 with a 1:1 stoichiometry at its heme group. This side of the heme is referred to as distal, the other as proximal. More than 40 years ago, it was proposed that O_2-binding involves the side-chain of the distal histidine 64 at the E7 helical position (Perutz and Mathews, 1966). Beside this 'histidine-gate' hypothesis, it was also shown that ligands may escape through the interior of the protein

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where hydrophobic Xe-binding cavities are observed (Tilton et al., 1984). Ligand binding to Mb then follows a multi-state scheme where internal states and distal cavities play a major role (Fig. 1-a) (Ostermann et al., 2000). Here, B states represent a number of ligand positions in the distal 'pocket' with differing rates of binding to the heme group. In the C state, the ligand is relocating between multiple states such as Xe1 and Xe2 cavities within the protein matrix (Scott et al., 2001). The current view is that the majority of ligands (~70–80%) enter and exit from the distal histidine gate (Scott et al., 2001; Elber, 2010) and thus, the kinetics is adequately interpreted in terms of an effective two-step scheme (Fig. 1-b).

Much insight into the pathways and kinetics of ligand binding to Mb has been obtained from studies of its site-directed mutants (Varadarajan et al., 1985; Springer and Sligar, 1987). By measuring the O_2 binding parameters of 90 sperm whale Mb mutants at 27 different positions, a mapping of the pathways for O_2 entry and exit was achieved (Scott et al., 2001). These investigations show that His E7 stabilizes bound O_2 about 1000-fold, most likely because of the formation of a strong hydrogen bond between O and $N_\varepsilon H$ of the imidazole group (Olson, 2008).

In general, the molecular evolution of the globin superfamily can be traced back ~4000 Myr to a common ancestor, which was among the basic protein components required for life (Suzuki and Imai, 1998; Wajcman et al., 2009). Mammalian Mb is thought to have diverged from another common ancestor about 600 Myr ago at the beginning of Cambrian period, and sequence alignments reveal a highly conserved distal pocket within all of these (Romero-Herrera et al., 1973).

The molecular evolution of the heme group can be partly rationalized through the positive selection of the porphyrin structure to satisfy the reversible spin-crossover upon O_2 binding to porphyrin, which is a necessary first condition for reversible binding of molecular O_2 to Mb (Jensen and Ryde, 2004). In this work, we investigate how the physiological function is further refined by the properties of the surrounding protein, *i.e.* the sequence-dependent thermochemistry of O_2-binding, by putting these data into a physiological framework and changing the physiological variables of O_2 partial pressure (P_{O2}), cell size, Mb concentration, and metabolic rate.

To this end, we quantify transport and storage proficiency as functions of physiological variables in the same way that "reversible binding" and "near-degeneracy" of spin states were used to quantify the functional proficiency of porphyrin and iron, specifically (Jensen and Ryde, 2003, 2004). We then investigate why and how selection pressure conserves the wild-type Mb. It is found that the relative importance of storage and transport varies from one mutant to another and is greatly affected by physiological and environmental conditions, and that the WT appears to have been selected under hypoxic conditions where other mutants are much less proficient.

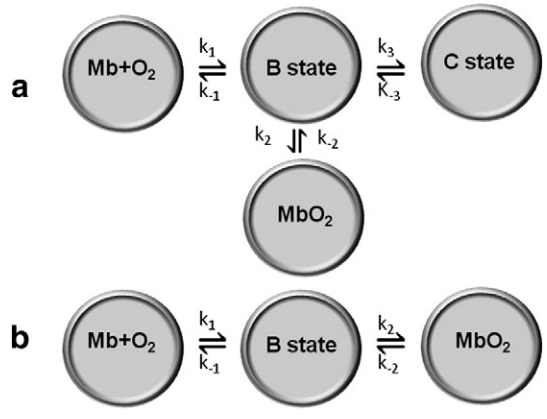

Fig. 1. Kinetics of O_2 binding to Mb. a) The C state accounts for internal cavities while in b) mainly the B state is involved under physiological conditions.

2. Theoretical background

2.1. Saturation expression

The saturation of Mb by O_2 (S), can be expressed in terms of the Hill equation (Hill, 1936):

$$S = \frac{\left(P_{O_2}\right)^n}{(P_{50})^n + \left(P_{O_2}\right)^n} \tag{1}$$

where P_{O_2} is the oxygen partial pressure, n is the oxygen binding cooperativity index, and P_{50} is the value of P_{O_2} at which $S = 0.5$. For Mb with only one subunit, $n = 1$ and saturation can be described using the bimolecular oxygenation equilibrium:

$$Mb + O_2 \rightleftharpoons MbO_2 \tag{2}$$

$$S = \frac{[MbO_2]}{C_{Mb}} = \frac{K_{O_2}[O_2]}{K_{O_2}[O_2] + 1}. \tag{3}$$

Here, saturation is defined as $S = \frac{[MbO_2]}{C_{Mb}}$, $K_{O_2} = \frac{[MbO_2]}{[O_2][Mb]}$ is the bimolecular oxygenation equilibrium constant, and C_{Mb} is the total concentration of Mb in the cell, which is equal to the sum of [Mb] and [MbO_2]. Eq. (3) can be converted to (1) using $P_{50} = \frac{1}{K_{O_2}\alpha_{O_2}}$ and $[O_2] = \propto_{O_2} P_{O_2}$, where α_{O_2} is the oxygen solubility constant in the sarcoplasm. In the general case of two-step binding (Fig. 1-b), using the equilibrium constants K_1 and K_2, S takes the form:

$$S = \frac{K_1K_2[O_2]}{K_1K_2[O_2] + K_1[O_2] + 1} = \frac{K_{O_2}\alpha_{O_2}P_{O_2}}{\alpha_{O_2}P_{O_2}\left(K_{O_2} + K_1\right) + 1}. \tag{4}$$

The second equality in Eq. (4) holds by using $K_{O_2} = K_1K_2$ and $[O_2] = \alpha_{O_2}P_{O_2}$, and the half saturation pressure can be calculated as $P_{50} = \frac{1}{\alpha_{O_2}(K_{O_2} - K_1)}$. For all mutants studied here $K_{O_2} \gg K_1$ and Eq. (4) reduces to Eq. (3), but this may not always be the case.

2.2. Muscle tissue and oxygen delivery

To model oxygen delivery within the muscle cell, we applied the Krogh cylinder model in a revised, state-of-the-art form (Groebe, 1995). As shown in Fig. 2, the model consists of three concentric cylinders representing *capillaries* (inner region), space between red blood cells and sarcolemma, or *cell free regions* (middle), and the *muscle tissue* as the outer region. We consider only radial oxygen diffusion and assume chemical equilibrium at all times, since both the radial and longitudinal diffusion coefficients of Mb are similar within our scope (Groebe, 1995; Lin et al., 2007b). The radial distance (r) is used as a generalized coordinate. With this theoretical framework, P_{O_2} is derived as:

$$P_{O_2}(r) = \frac{1}{2}\left(P^*(r) - \frac{D_{Mb}C_{Mb}}{D_{O_2}\alpha_{O_2}} - P_{50}\right) + \frac{1}{2}\sqrt{\left(P^*(r) - \frac{D_{Mb}C_{Mb}}{D_{O_2}\alpha_{O_2}} - P_{50}\right)^2 + 4P^*(r)P_{50}} \tag{5}$$

where $D_{O_2}\,(m^2\,s^{-1})$ and $D_{Mb}\,(m^2\,s^{-1})$ are the free oxygen and Mb diffusion coefficients in the cell, $C_{Mb}\,(mol\ L^{-1})$ and $\alpha_{O_2}\,(mol\ L^{-1}\,mm\,Hg^{-1})$ are the total concentration of Mb and the oxygen solubility in muscle tissue.