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Myoglobin extraction from mammalian skeletal muscle and oxygen affinity determination under physiological conditions

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ABSTRACT

An accurate determination of myoglobin (Mb) oxygen affinity (P_{50}) can be difficult due to hemoglobin (Hb) contamination and autoxidation of Mb to metMb which is incapable of binding oxygen. To reduce Mb autoxidation, P_{50} is often measured at refrigerated temperatures. However, the temperature dependent shift in Mb oxygen affinity results in a greater oxygen affinity (lower P_{50}) at colder temperatures than occurs at physiological temperature (ca. 37–39 °C) for birds and mammals. Utilizing the temperature dependent pH shift of Tris buffer, we developed novel methods to extract Mb from vertebrate muscle samples and remove Hb contamination while minimizing globin autoxidation. Cow (*Bos taurus*) muscle tissue ($n = 5$) was homogenized in buffer to form a Mb solution, and Hb contamination was removed using anion exchange chromatography. A TCS Hemox Blood Analyzer was then used to quickly generate an oxygen dissociation curve for the extracted Mb. The oxygen affinity of extracted bovine Mb was compared to commercially available horse heart Mb. The oxygen affinity of extracted cow Mb ($P_{50} = 3.72 \pm 0.16$ mmHg) was not statistically different from commercially prepared horse heart Mb ($P_{50} = 3.71 \pm 0.10$ mmHg). With high yield Mb extraction and fast generation of an oxygen dissociation curve, it was possible to consistently determine Mb P_{50} under physiologically relevant conditions for endothermic vertebrates.

Introduction

Myoglobin (Mb)[2] is a globular hemoprotein found in vertebrate cardiac and skeletal muscle that reversibly binds oxygen. In muscle cells, Mb buffers mitochondrial oxygen availability, facilitates oxygen diffusion, and at high concentrations found in the muscle of many diving birds and mammals, can act as a significant oxygen store to maintain aerobic metabolism during hypoxia (for review see: [1–4]). Mb was the first protein to have its three dimensional structure rendered [5,6], and it remains a model for studying the relationship between protein structure and function [7–9]. Although Mb is one of the most studied of all proteins, much of the research has focused on its protein chemistry rather than its function under physiological conditions.

Hemoglobin (Hb) is a similar globular hemoprotein capable of reversibly binding oxygen that is expressed in vertebrate red blood cells. Oxygen binding respiratory pigments such as Mb and Hb are characterized by the affinity with which they bind oxygen. This oxygen affinity is quantified as the partial pressure of oxygen at which 50% of pigments in solution are bound with oxygen (P_{50}). The P_{50} of these respiratory pigments can be determined by generating an oxygen dissociation curve (ODC) (Fig. 1). These orthologous globins share much of their overall structure (characteristic "globin fold"), and many key regions are highly conserved [10]. With similar structure and heme binding properties, the optical characteristics of these proteins are similar [11,12] (Figs. 2 and 3) and these hemoproteins cannot be distinguished by spectroscopy when in solution together [11]. At wavelengths of 500–700 nm, the absorption spectra of HbO_2 and MbO_2 both show twin absorption peaks: 544 and 582 nm for MbO_2 and 542 and 578 nm for HbO_2. In the deoxygenated state, Mb has a single peak at 557 nm and Hb at 554 nm. (For Mb see: [13–15]. For Hb see: [16–18]). Because the optical properties in the oxygenated and deoxygenated state are similar, these globins exhibit nearly identical spectral shifts during oxygen binding and dissociation from 500 to 700 nm. This shift can be used to monitor the oxygen binding state

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[2] *Abbreviations used:* Hb, hemoglobin; Mb, myoglobin; ODC, oxygen dissociation curve.

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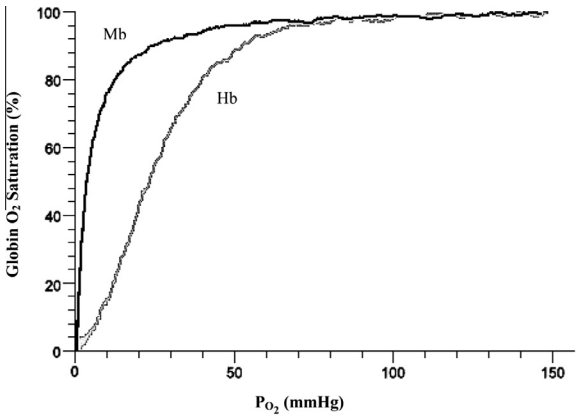

Fig. 1. Oxygen dissociation curves of horse heart myoglobin (P_{50} = 3.70) and human hemoglobin (P_{50} = 23.64) determined using TCS Hemox Blood Analyzer.

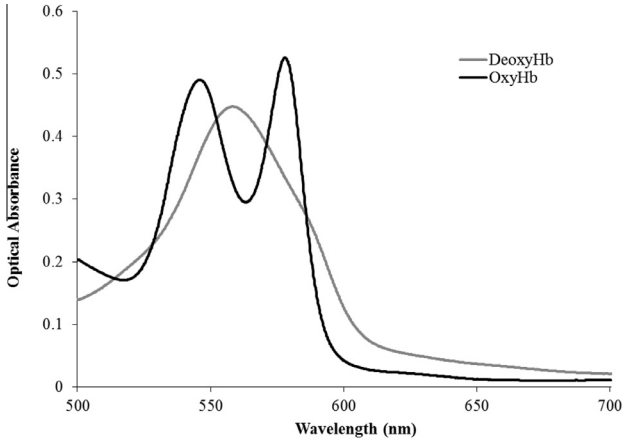

Fig. 2. Spectral scan of human oxyhemoglobin and deoxyhemoglobin from 500 to 700 nm wavelength.

of these pigments in solution, and due to their nearly identical optical responses, identical instrumentation can be used.

The functional properties of Mb are highly dependent on protein structure, and mutational studies have examined the effects of amino acid substitutions on Mb affinity for oxygen [19–22] and other ligands [23]. However, relatively few studies (24, 25, 26) have compared the oxygen affinity of naturally occurring Mb structural variants for different vertebrate species using identical experimental methods. Although Mb oxygen affinities have been reported for a variety of species in the literature, the studies used varying experimental techniques, instrumentation, and temperatures, making interpretation of the data for comparative analyses difficult. Extracting Mb from muscle to measure oxygen affinity at physiological temperatures (ca. 37–39 °C) for mammals and birds poses two challenges: (1) endogenous Hb is an unavoidable contaminant when homogenizing muscle samples, and (2) Mb in solution quickly autoxidizes to non-functioning metMb at physiological temperatures [27]. Because Mb and Hb have similar optical properties but very different oxygen affinities, Hb is a significant contaminant that can alter the photometric Mb P_{50} and may constitute as much as 30% of the total globin content of excised skeletal muscle in beef cattle [28,29]. While reducing agents such as dithionite may be used to chemically reduce Mb in solution, they are then typically removed by buffer exchange before experimentation [24,27,30].

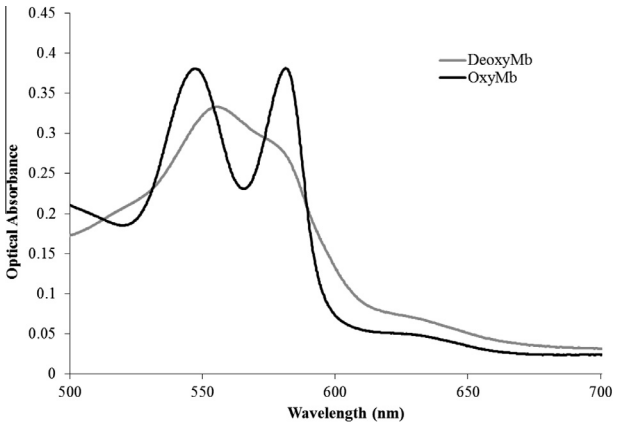

Fig. 3. Spectral scan of horse oxymyoglobin and deoxymyoglobin from 500 to 700 nm wavelength.

The autoxidation rate of Mb to inactive metMb is slowed under conditions of low temperature and alkaline pH [31,32]. To minimize the autoxidation of Mb in solution, P_{50} measurements are often made at temperatures that are much lower than the core body temperature of mammals and birds. However, changes in temperature affect the rate of ligand dissociation from Mb disproportionately to the association rate resulting in a pronounced increase in Mb oxygen affinity with reduced temperature [33]. Because of this temperature dependent shift in Mb oxygen affinity, measurements at reduced temperature are not directly physiologically relevant [27], but it is possible to estimate a physiologically significant P_{50} after determining oxygen affinity at multiple temperatures and applying a compensatory factor to correct for in vivo muscle temperature [25,27]. In living tissue, oxidized metMb is returned to its active form by the enzyme metMb reductase. While this enzyme is present and active in muscle homogenates, its concentration is greatly reduced during Mb purification [34].

As part of a larger study comparing the oxygen affinity of Mb among a variety of endothermic vertebrates, we developed a simplified method for extracting Mb from vertebrate muscle. Our goal was to develop a simplified, uniform method to extract Mb from vertebrate skeletal muscle and determine Mb oxygen affinity. Using purification methods that remove Hb contamination while minimizing Mb oxidation, we eliminated the need for reducing agents (e.g. dithionite) that must be removed by buffer exchange before measuring oxygen affinity.

Materials and methods

Buffer selection

To maximize the yield of functional Mb during extraction and purification, we used procedures that minimized processing time and maintained a low temperature (0–4 °C) and high pH (>8) (see Appendix for stepwise methods). A single stock buffer solution of 50 mM Tris buffer (Sigma–Aldrich, T 0694) with the addition of 50 mg l^{-1} gentamicin sulfate (Sigma–Aldrich, G-1264) to prevent microbial contamination was used throughout tissue homogenization and purification. While Tris buffers are notorious for temperature dependent shifts in pH, we took advantage of this property to prepare and analyze samples using a single buffer solution. The temperature dependent shift in pH made it possible to chromatographically remove Hb from muscle homogenates and subsequently generate an ODC at a physiologically relevant temperature and pH using one buffer (pH = 7.4 at 37 °C; pH = 8.26 at 5 °C). This eliminated the need for buffer exchange