Analytical Biochemistry 400 (2010) 33–45

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

# A quantitative method to assess muscle tissue oxygenation in vivo by monitoring $^1$H nuclear magnetic resonance myoglobin resonances

Maristella Gussoni [a,*], Mauro A. Cremonini [b], Alessandra Vezzoli [c], Fulvia Greco [d], Lucia Zetta [d]

[a] Dipartimento di Scienze e Tecnologie Biomediche, Università di Milano, Segrate, Milano 20090, Italy
[b] Dipartimento di Scienze degli Alimenti, Università di Bologna, Cesena 47023, Italy
[c] Istituto di Bioimmagini e di Fisiologia Molecolare, CNR, Segrate, Milano 20090, Italy
[d] Istituto per lo Studio delle Macromolecole, CNR Lab. NMR, Milano 20133, Italy

ARTICLE INFO

*Article history:*
Received 4 September 2009
Received in revised form 10 December 2009
Accepted 14 January 2010
Available online 21 January 2010

*Keywords:*
$^1$H NMR
Myoglobin
Tissue oxygenation
In vivo
*Arenicola marina*

ABSTRACT

A nuclear magnetic resonance (NMR) method was implemented to assess in vivo oxygenation levels by a quantitative determination of the $^1$H NMR myoglobin (Mb) resonances. The proximal His-F8 $N_\delta H$ at 70–90 ppm and Val-E11 $\gamma CH_3$ resonance at –2.8 ppm, reflecting deoxygenated (deoxy-Mb) and oxygenated (met-Mb) states, were alternately recorded. The method was developed in vitro choosing a couple of NMR sequences that could each maximize the signal-to-noise ratio (*SNR*) while avoiding baseline rolling and suppressing the water signal. Two quantitative calibration methods were implemented for deoxy- and met-Mb samples (0.1–1 mM), respectively. The respective limit of detection (LOD) and limit of quantification (LOQ) were 0.015 and 0.05 mM for met-Mb and 0.013 and 0.042 mM for deoxy-Mb. Sequences and calibration curves were employed in vivo in *Arenicola marina* to obtain, for the first time, an accurate measurement of oxy- and deoxy-Mb actual concentrations. In *Arenicola,* the peaks at approximately 87 and –2.7 ppm, reflecting the deoxy- and oxy-Mb states, respectively, were alternately recorded during increasing hypoxia. The deoxy-Mb concentrations were obtained from the calibration curve. The oxy-Mb concentrations were calculated from the calibration of met-Mb because it was proved that oxy- and met-Mb gave the same NMR molar response. From oxy- and deoxy-Mb concentrations, the intracellular oxygen partial pressure ($P_iO_2$) trend was determined.



Oxygen plays various roles in cells, usually strictly dependent on its concentration. Because many factors can affect both oxygen delivery and oxygen use, the most physiologically pertinent measurements of tissue oxygenation are those performed in vivo by means of direct methods for measuring oxygen partial pressure ($PO_2$)[1] in cells and tissues. Consequently, many techniques have been developed for measuring, both qualitatively and quantitatively, oxygen availability in vivo used as researching tools and also in the clinical setting. Each of them offers peculiar potential advantages and limitations so that the use is complementary rather than competitive.

All methods have been reported and reviewed extensively, including non-magnetic resonance (MR)- and MR-based techniques [1–3]. Among those belonging to the first category, polarographic needle oxygen electrodes have long been considered as a "gold standard" in clinical use for measuring both baseline and dynamic $PO_2$ response levels even if the method suffers from signal-to-noise problems at low oxygen tension [2]. Fluorescent and phosphorescent quenching methods are also commercial systems that involve the insertion of fiber-optic oxygen sensors into tissues to directly measure oxygenation and, like the electrode methods, are more or less invasive and time-consuming [3]. Other noninvasive techniques, in particular near-infrared spectroscopy (NIRS), offer many practical advantages such as high temporal resolution, clinical applicability, favorable cost effectiveness, and portability. However, NIRS provides data indirectly, raising questions concerning the compartment of the measurements, so that the method does not return an actual estimate of the tissue $PO_2$ [4].

By contrast, MR-based methods have the potential to noninvasively investigate parameters related to $O_2$ metabolism and to well

* Corresponding author. Fax: +39 023699610.
*E-mail address:* maristella.gussoni@unimi.it (M. Gussoni).

[1] *Abbreviations used:* $PO_2$, oxygen partial pressure; MR, magnetic resonance; NIRS, near-infrared spectroscopy; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; PFC, perfluorocarbon; Mb, myoglobin; deoxy-Mb, deoxygenated Mb; TSP, sodium-3-trimethylsilylpropionate; oxy-Mb, oxygenated Mb; met-Mb, Mb bearing a high spin $Fe^{3+}$ ion; NO, nitric oxide; $[PO_2]_{50}$, oxygen partial pressure at myoglobin half-saturation; *SNR,* signal-to-noise ratio; $P_iO_2$, intracellular oxygen partial pressure; PBS, potassium phosphate-buffered solution; SDT, sodium dithionite; *NS,* number of scans; *RG,* receiver gain; *TR,* repetition time; *DE,* pre-scan delay between the radiofrequency pulse and the start of acquisition; FID, free induction decay; LOD, limit of detection; LOQ, limit of quantification; *TE,* echo time; SD, standard deviation.

0003-2697/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2010.01.022

discriminate vascular and intracellular oxygenation. The techniques include nuclear magnetic resonance (NMR) spectroscopy and imaging as well as electron paramagnetic resonance (EPR) and related methods, such as dynamic nuclear polarization, that combine aspects of EPR and NMR. EPR oxymetry provides a potentially very useful method to follow changes in $PO_2$ under various physiological and pathological conditions, in particular giving a peculiar contribution to the possibility of the development of novel approaches for modifying tumor oxygenation, thereby optimizing anticancer treatments. However, the technique does not allow direct detection of $O_2$ owing to the dramatic broadening of EPR lines in biological systems. At the same time, the lack of detectable levels of endogenous paramagnetic species makes it necessary to use exogenous spin probes endowed with unpaired electrons such as nitroxides and triarylmethyl radicals [3].

On the other hand, the indirect detection of oxygen by NMR can be performed by observing the effect of the $O_2$ paramagnetism on the NMR signals via the alteration of the relaxation times of the detected nuclei. For clinical applications, in particular tumor detection, $^1$H and even more $^{19}$F have been used. Indeed, $^{19}$F NMR spectroscopy and imaging of perfluorocarbon (PFC) emulsions have been used extensively as a sensitive indicator of tumor oxygenation [5].

Moreover, NMR methods have the widely recognized potential to noninvasively investigate parameters related to $O_2$ metabolism and, in particular, to well discriminate vascular and intracellular oxygenation. Several methods can be adopted to evaluate tissue perfusion and oxygenation in different organs, specifically in the skeletal muscle of humans and animal models [6,7].

Furthermore, NMR spectroscopy can, in a noninvasive and nondestructive manner, localize metabolite signals in tissues, revealing their changes under different physiological and pathological conditions. In particular, when muscle is the target of investigation, $^1$H lactate and $^{31}$P phosphate resonances offer a quantitative assessment of the anaerobic metabolism [8,9] and can also be regarded as *indirect* indicators of the cellular oxygenation levels. Nevertheless, $^1$H NMR of myoglobin (Mb) is widely considered as a *direct* method to assess intramyocitic oxygenation in skeletal and heart muscle tissues [10], in particular under conditions characterized by low oxygen levels such as exercise and ischemia [11–13].

Mb, an oxygen binding protein belonging to the globin superfamily, plays an essential role in maintaining aerobic metabolism. Both its restricted distribution to endurance muscle and heart cells throughout the vertebrates and its notable expression in diving mammals reflect its widely accepted function in cellular oxygen transport and oxygen buffering. That function is to support high levels of aerobic muscular activity as an intracellular source of oxygen during periods of reduced supply by blood and as a molecule facilitating the $O_2$ transfer from sarcolemma to mitochondria, in particular during extended periods of hypoxic physiological conditions such as muscle tissue during heavy work [14].

The proton resonances of cytosolic Mb offer a unique opportunity to assess intracellular muscular oxygenation because the heme iron exhibits electronic structure changes depending on oxygen amount. In fact, when the oxygen concentration is low, Mb is principally in the deoxygenated state (deoxy-Mb); the heme $Fe^{2+}$ electrons are unpaired ($S = 2$) and deoxy-Mb is paramagnetic [15]. The electron interaction with the proximal His-F8 $N_\delta H$ produces a hyperfine shifted peak in the 70 to 90 ppm $^1$H NMR spectral region, downfield from sodium-3-trimethylsilylpropionate (TSP), that exhibits a temperature-dependent chemical shift [16,17]. On the contrary, on oxygenation, the heme iron is ligated with oxygen (oxy-Mb) and the $Fe^{2+}$ electrons are paired ($S = 0$) and oxy-Mb is diamagnetic. The amino acid residues close to the heme undergo the effect of local magnetic fields, shifting their resonance positions. In particular, the Val-E11 $\gamma CH_3$, located on the distal side of the heme, experiences a ring current that induces an upfield shift to approximately –2.8 ppm from TSP [18,19].

On nitrite oxidation, the characteristic set of met-Mb (Mb bearing a high spin $Fe^{3+}$ ion) peaks appear in the $^1$H NMR spectra; the Val-E11 $\gamma CH_3$ and $\gamma'CH_3$ resonances are paramagnetically shifted to –3.7 and –4.7 ppm, respectively [20]. These signals could also be recorded in vivo, and a possible (albeit controversial) role of Mb as bioactive nitric oxide (NO) producer and scavenger was postulated [21–24].

The quantitative determination of oxy- and deoxy-Mb $^1$H NMR signals in muscle tissue provides a method for directly calculating the $PO_2$ in heart and skeletal muscles, starting from the Mb oxygen binding equation: $K = 1/[PO_2]_{50} = [\text{oxy-Mb}]/[\text{deoxy-Mb}]\ PO_2$, where oxy-Mb and deoxy-Mb concentrations are obtained from the correspondent NMR peak areas and the values for $[PO_2]_{50}$ (i.e., partial pressure of oxygen that will half-saturate the Mb) are available in the literature [25]. The equation can be rearranged as

$$PO_2 = [\text{oxy-Mb}]/[\text{deoxy-Mb}] \times [PO_2]_{50}, \quad (1)$$

thereby establishing a simple direct method to assess muscle intracellular oxygenation as an alternative to normally adopted procedures. In spite of this, in several studies on humans, skeletal muscle oxygenation was evaluated through the measurement of the paramagnetic deoxy-Mb resonance only [12,26–28], giving rise to a quantitative determination problem because neither [oxy-Mb] nor the total Mb concentration was known.

Oxy-Mb was previously detected mainly in ex vivo models, such as excised perfused heart [19], using the binomial 1331 pulse sequence to excite the Mb resonances while suppressing the water signal [29]. However, excitation with the 1331 sequence led to large phase dispersion, resulting in positive lipid signals and negative Val-E11 methyl oxy-Mb and met-Mb resonances [22,30]. Owing to the phase and baseline corrections that needed to be applied, the resulting data were quantitatively biased [19], and this is inconvenient for analytical purposes.

On the basis of such considerations, the current study aimed at implementing an NMR method to assess tissue muscle oxygenation levels by means of an accurate quantitative determination of the proximal His-F8 $N_\delta H$ and Val-E11 $\gamma CH_3$ resonances, probe of Mb in the deoxygenated and oxygenated states, respectively, and recorded in alternate experiments in the same sample. This was obtained by first choosing in vitro a couple of NMR sequences that could each maximize the signal-to-noise ratio (*SNR*) of the oxy- and deoxy-Mb NMR signals while avoiding baseline rolling and still sufficiently suppressing the water signal. Then an external calibration curve was built from a number of deoxy- and met-Mb solution samples. Finally, these sequences and calibration curves were employed to estimate, for the first time, the actual concentrations of both forms of bioactive Mb under increasing hypoxia and calculate the intracellular $PO_2$ ($P_iO_2$) in *Arenicola marina,* a hypoxia-tolerant lungworm widely used as a living model to study metabolic adaptation in oxy-conformer tissues [31].

## Materials and methods

### *Test samples*

Horse skeletal muscle Mb in the ferric form (met-Mb) was purchased from Sigma Chemical as lyophilized salt-free powder and used without further purification.

Test samples were freshly prepared by dissolving met-Mb (in the range of 0.1–1 mM concentration) in aqueous 100 mM potassium phosphate-buffered solution (PBS) at pH 7.4. $^2H_2O$ (10% final concentration) was added for lock and shim, and 750 μl of the final solution was put in a 5-mm NMR tube.