Detection of myoglobin desaturation in *Mirounga angustirostris* during apnea

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Ponganis, Paul J., Ulrike Kreutzer, Napapon Sailasuta, Torre Knower, Ralph Hurd, and Thomas Jue. Detection of myoglobin desaturation in *Mirounga angustirostris* during apnea. Am J Physiol Regulatory Integrative Comp Physiol 282: R267–R272, 2002; 10.1152/ajpregu.00240.2001. — 1H NMR solution-state study of elephant seal (*Mirounga angustirostris*) myoglobin (Mb) and hemoglobin (Hb) establishes the temperature-dependent chemical shifts of the proximal histidyl N, H signal, which reflects the respective intracellular and vascular PO2 in vivo. Both proteins exist predominantly in one major isoform and do not exhibit any conformational heterogeneity. The Mb and Hb signals are detectable in *M. angustirostris* tissue in vivo. During eupnea *M. angustirostris* muscle maintains a well-saturated MbO2. However, during apnea, the deoxymyoglobin proximal histidyl N, H signal becomes visible, reflecting a declining tissue PO2. The study establishes a firm methodological basis for using NMR to investigate the metabolic responses during sleep apnea of the elephant seal and to secure insights into oxygen regulation in diving mammals.

nuclear magnetic resonance; oxygen; hypoxia; seal; apnea; eupnea

MYOGLOBIN (Mb) is often postulated to play a physiological role in supplying O2 to muscle during both hypoxemia and ischemia. The role of Mb as an O2 store arises in part from observations that diving mammals have higher concentrations of Mb than terrestrial mammals and in part from the depletion of Mb O2 in seals during the severe bradycardia, muscle ischemia, and progressive hypoxemia in forced submersion experiments. In young harbor seals (*Phoca vitulina*), for example, complete depletion of Mb O2 and an increase in lactate production occur within 10 min during forced submersion experiments (33).

However, free-diving studies have suggested that the physiological response during a natural dive can differ sharply from the response during a forced submersion (4, 13). In contrast to the forced submersion findings, bradycardia is less intense in free-diving Weddell seals (*Leptonychotes weddellii*) (11), postdive lactate accumulation does not begin until after dives of 19-min duration (13), and Mb desaturation appears to be incomplete even after dives of 30 min (10). These observations raise questions about MbO2 depletion during a free dive and the function of Mb, either as a store of O2 or as a facilitator of O2 diffusion (10, 12, 32, 33, 36).

Sleep apnea in elephant seals (*Mirounga angustirostris*) represents a unique model to investigate the function of Mb during a breath hold (apnea) in a diving mammal. During sleep, these animals exhibit routine prolonged apneas interrupted by intermittent ventilatory periods (eupneas). In fact, Northern elephant seals (*M. angustirostris*) undergo several cycles of apnea and eupnea within a single slow-wave sleep episode (6). Mild bradycardias and constant lactate concentrations are associated with these breath holds (1, 5, 6). These physiological responses suggest that cardiovascular and metabolic responses during sleep apnea are more similar to those during free diving than forced submersion. Sleep apnea, therefore, provides an opportunity to examine the interplay of vascular O2 delivery, Mb function, and cellular metabolic responses during the progressive hypoxemia and probable muscle ischemia during the breath hold.

Although recent near-infrared spectroscopy methods have now measured a decrease in the composite Mb and hemoglobin (Hb) signal in Weddell seals during a free dive, they cannot distinguish the relative vascular vs. cellular contribution (10). 1H NMR techniques, however, can detect noninvasively both the deoxymyoglobin (deoxyMb) and deoxyhemoglobin (deoxyHb) proximal histidyl N, H signals in vivo. These signals increase in intensity as the O2 level drops (14, 35). Rat myocardium studies have substantiated the methodology and have further demonstrated that the Mb valine E11 γCH3 signal at –2.8 parts per million (ppm), whose signal intensity mirrors the cellular oxygenated state, is also observable. The deoxyMb and deoxyHb peaks yield then a quantitative measurement of the intracellular and vascular oxygenation (15, 16, 18, 35).

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The present report establishes the experimental basis for implementing a $^1$H NMR strategy to follow MbO$_2$ and HbO$_2$ desaturation in seal muscle during apnea. The $^1$H NMR solution spectra of both deoxyMb and deoxyHb from the elephant seal exhibit the characteristic proximal histidyl N$_2$H peaks in the region between 60 and 100 ppm and do not reveal significant presence of other isozymes, which can vary from species to species (14, 17). The proximal histidyl N$_2$H NMR signals in the paramagnetic state of deoxyMb and deoxyHb exhibit the interaction between the unpaired Fe electrons and the nuclear spin, usually termed as the hyperfine interaction, which exhibits a characteristic relationship between chemical shift and 1/T, where T is temperature (19). Indeed, the $^1$H NMR technique detects MbO$_2$ desaturation and resaturation during apnea and eupnea in the elephant seal. The report then sets the experimental basis for using $^1$H NMR to investigate O$_2$ regulation and the MbO$_2$ response during apnea, which can yield unique insights into the physiological dynamics during a voluntary breath hold.

**MATERIALS AND METHODS**

**Mb Purification**

*M. angustirostris* Mb was obtained from a juvenile elephant seal carcass that had been preserved at $-20^\circ$C after death from presumed natural causes at Ano Nuevo Island. A sample of muscle was frozen in liquid nitrogen, and the Mb was prepared in accordance with previously reported procedures (37).

The tissue was ground to a fine powder under liquid nitrogen, homogenized in 3 vol of distilled H$_2$O, centrifuged 1 h at 16,000 g, and filtered through cheesecloth. All biochemical preparation procedures were carried out at 4°C. After two ammonium sulfate precipitation steps, 0–70% and 70–100% saturation, each followed by centrifugation, the pellet of the second precipitation was dissolved in 5 mM Tris buffer (pH 8.4), dialyzed against the same buffer, and loaded on a DEAE ion-exchange column. The column was washed with three volume columns of 5 mM Tris (pH 8.4). Mb was eluted with 50 mM Tris (pH 8.4). Samples were analyzed spectrophotometrically at 581 nm (the $\alpha$-band of MbO$_2$).

Mb-containing fractions were pooled and concentrated by ultrafiltration. SDS-PAGE with 17.5% gels using a Tris-glycine buffer revealed a single protein band corresponding to $\sim$16,000 Da compared with calibration markers. Gels were stained with Coomassie brilliant blue.

Mb concentration was determined spectrophotometrically using published absorption coefficients for horse oxymyoglobin (oxyMb) (2). Purity was checked electrophoretically. Mb samples were stored as metcyanomyoglobin (metMbCN). MbO$_2$ was converted to metMbCN by addition of three times excess KCN and K$_3$Fe(III)(CN)$_6$. Samples were then treated by gel filtration on PD$_{10}$ columns (Pharmacia) equilibrated with 100 mM potassium phosphate and 2 mM KCN (pH 7.4) to remove excess K$_3$Fe(III)(CN)$_6$. The metMbCN was lyophilized and stored at $-80^\circ$C.

**Hb Purification**

Blood was obtained from the extradural vein of a sedated (2 mg/kg im ketamine) captive juvenile elephant seal at Scripps Institution of Oceanography (SIO). Blood was collected in 10-ml vacutainer tubes with 500 U of heparin and immediately shipped on ice to the University of California, Davis. Hb was prepared following a previously reported procedure (2). *M. angustirostris* blood was centrifuged 10 min at 600 g and washed three times with 1% NaCl. Erythrocytes were lysed with 3 vol of distilled H$_2$O. The lysate was centrifuged 30 min at 30,000 g, and the supernatant was fractionated by ammonium sulfate precipitation (0–20% saturation). The sample was centrifuged 30 min at 10,000 g, and the supernatant was dialyzed against 10 mM potassium phosphate (pH 7.4). Hb was stored as HbCO and was converted to HbCN with a procedure similar to that used in preparing MbCN, as described in *Mb Purification*.

**Preparation of DeoxyMb and DeoxyHb**

Lyophilized metMbCN samples were dissolved in H$_2$O, and 0.5 ml was transferred into a Centrifor 10 (Millipore) microconcentrator; several exchanges of buffer through four to five concentration-dilution cycles achieved the final ionic strength and pH. The sample was then introduced into a 5-mm NMR tube, gassed with N$_2$, and sealed with a rubber stopper. A slight stoichiometric excess of freshly prepared sodium dithionite was injected through the stopper septum to form deoxyMb. For deoxyHb, the CO was first photodissociated under a stream of O$_2$ gas.

**NMR Spectroscopy**

Isolated protein. $^1$H NMR spectra of isolated Mb and Hb samples were collected on a Bruker AM 400 NMR spectrometer equipped with a 5-mm $^1$H observe/$^{13}$C decouple probe. The H$_2$O resonance was reduced by a water presaturation pulse. The 90° pulse was calibrated against the residual water line. The total repetition time for each scan was 200 ms. Spectral width was set at 50–90 ppm, with 4K data points; 2,000 scans were accumulated for each spectrum. All peaks were referenced to the water signal at 4.76 ppm (25°C), calibrated against 2–2-dimethyl-2-silapentane-5-sulfonate (DSS) as 0 ppm. For temperature studies, the variable temperature (VT) unit of the spectrometer was calibrated with ethylene glycol (30). Protein samples were incubated for 15 min at the respective temperature to allow for complete temperature equilibration. Before Fourier transformation, the free induction decays were multiplied by a 10-Hz exponential filter.

**In vivo measurement**

A 60-kg, 7-mo-old male elephant seal, obtained from the Sea World Rehabilitation Program and maintained at the SIO ring tank facility, was trained to rest prone, strapped in a wooden cradle fitted to the magnet bore. For the NMR study, the seal was first transported by van from San Diego, CA, to the University of California, Santa Cruz, where it was housed temporarily at the pinniped facility. It was later transported to the General Electric research facility at Fremont, CA.

In vivo NMR measurements were performed with a 1-m bore diameter GE Signa scanner at 1.5 T. During either the continuous acquisition of $^1$H or $^{31}$P signals, a trained observer in the magnet room monitored the seal’s breathing pattern and signaled to the investigators in the NMR control room the periods of eupnea/apnea, which were then referenced to the appropriate signal acquisition data block.

$^1$H (63.86 MHz) NMR signal acquisition used a body coil transmit/surface coil (5-in. diameter) receive configuration. The receive coil was positioned over the region of the longissimus dorsi muscle group. Magnetic field shimming was achieved using a three-point Dixon method, yielding a water line width about 40 Hz (8). A modified-DANTE pulse sequence excited the deoxyMb His-F8 N$_2$H signals, $\sim$4.6 kHz...
from the water resonance (29). Each spectrum required 200 transients, which corresponds to a total acquisition time of 40 s.

$^{31}$P (25.85 MHz) signal acquisition used a conforming flexible coil, which was wrapped around the seal covering the $^1$H receive coil. Each $^{31}$P NMR spectrum consisted of 25 transients and required a total acquisition time of 70 s.

Data were imported from the Signa system to a Sun Sparc2 workstation and processed using Omega 6.0 software. All spectra were zero filled to 2 K and apodized using a Gaussian-exponential function with a line broadening of 50 Hz. All spectra were baseline corrected and referenced to water at 4.65 ppm.

RESULTS

Figure 1 shows the $^1$H solution spectra from the native metMbCN and metcyanohemoglobin (MetHbCN) of *M. angustirostris*. In the MbCN spectrum, the major peaks at 27.4, 18.4, and 13.1 ppm are consistent with peak assignment to the heme methyls, as established in previous spectral analysis (24). In the HbCN spectrum, the major peaks at 21.64, 16.69, and 15.54 ppm also correspond to the heme methyls. The composite peak at 21.64 ppm originates from the five-heme methyl group from both the $\alpha$- and $\beta$-subunits (25). The peak also has an unassigned upfield shoulder, arising presumably from another heme group. Neither the *M. angustirostris* Mb nor Hb spectra reveal any spectral features or additional peaks that would indicate the presence of any heme disorder in the native protein, as observed in native yellow fin tuna muscle (27). Both electrophoresis and NMR analyses confirm one dominant isoform.

Figure 2 shows the $^1$H solution spectra from deoxyMb and deoxyHb in the region between 70 and 100 ppm. Both Mb and Hb exhibit the characteristic signals of the proximal histidine arising from the hyperfine interaction with the unpaired heme Fe electrons (9, 20). The temperature-dependent deoxyMb proximal histidyl N-H signal resonates at 76.0 ppm, while the corresponding signals from the $\alpha$- and $\beta$-subunits of Hb resonate at 61.8 and 73.2 ppm, respectively (27). At 25°C these signals shift to 78.5 (Mb), 76.0 ($\beta$-Hb), and 63.4 ppm ($\alpha$-Hb). Neither the Mb nor Hb spectra show any contaminating metmyoglobin or methemoglobin signals, and the chemical shifts are consistent with previously reported values (20, 21, 23). Figure 3 shows the typical chemical shift dependence on $1/T$ for a
The paramagnetic system (14, 23). The Mb and β-Hb signals maintain -3 ppm shift difference over the physiological temperature range.

The solution studies establish a firm basis for detecting the deoxyMb signal in *M. angustirostris* tissue and for determining tissue temperature. Figure 4 shows the 1H NMR spectrum acquired during a eupnea-apnea cycle. Apnea durations varied from 1 to 10 min in this animal. During eupnea, when the animal is breathing normally, NMR detects no tissue signal in the 50- to 90-ppm region. After 3 min of apnea, the 1H spectrum reveals the deoxyMb proximal histidyl NαH signals at 76 ppm (Fig. 4B). A slight upfield shoulder arises from the β-deoxyHb proximal histidyl NαH signal. The limited bandwidth of the selective pulse sequence does not significantly excite the α-deoxyHb proximal histidyl NαH resonance at 63 ppm. When the animal resumes normal breathing, the deoxyMb signal disappears rapidly (Fig. 4C).

Figure 5 shows the corresponding 31P spectra during a eupnea-apnea-eupnea cycle. The spectra show no significant perturbation of any high-energy phosphate signals during the eupnea-to-apnea transition.

**DISCUSSION**

*Mb and Hb Solution Spectra*

Consistent with the electrophoresis analysis, the distinct heme methyl resonances of the MbCN and HbCN spectra reveal one major isoform for *M. angustirostris* Mb as well as Hb. Any minor isoform component with a relative contribution of >5% would exhibit a distinct set of signals. None is detected in either the metcyanoMb/Hb or deoxyMb/Hb spectra. In addition, the seal Mb/Hb shows no conformational heterogeneity arising from heme inserted into the Mb protein in two different orientations (22, 27, 28). Any orientational heterogeneity would yield two sets of signals. In contrast, native Mb from vertebrate as well as invertebrate tissue often exhibits pronounced heme heterogeneity (17, 26, 27; Kreutzer and Jue, unpublished observations).

*DeoxyMb and DeoxyHb Spectra from M. angustirostris*

The applicability of the 1H NMR technique to detect the Mb signal in *M. angustirostris* tissue is clearly shown in the dynamic change of the proximal histidyl NαH signal during the eupnea-apnea cycle. Upfield of the deoxyMb signal appears the deoxyHb signal from the β-proximal histidyl NαH. These assignments are consistent with previously reported results from perfused myocardium and erythrocyte studies (15, 34). Clearly, the resting intracellular PO2 in seal skeletal muscle maintains a saturated MbO2 state, consistent with the observations of Mb in human gastrocnemius studies (35). During apnea, intracellular PO2 decreases.

The protein solution study has established the proximal histidyl NαH chemical shift positions for *M. angustirostris* deoxyMb and deoxyHb at different temperatures. Quite clearly, the chemical shifts of the Mb proximal histidyl NαH signals from seal and horse are similar, 78.5 and 78.9 ppm at 25°C, respectively. However, they differ from the corresponding signal from human Mb, which resonates at 80.3 ppm at 25°C. Such differences arise from the protein-heme interaction (7, 19).
At 35°C the deoxyMb proximal histidyl N₆H signal resonates at 76.0 ppm, while the corresponding signals from the α- and β-subunits of deoxyHb resonate at 61.8 and 73.2 ppm, respectively. The Mb spectral data indicate that during apnea the tissue temperature remains at ~35°C.

Mb Desaturation During Apnea

In M. angustirostris, Mb desaturates during apnea and returns to its normoxic level during eupnea. No deoxyMb signal is detected during the eupneic control state, which indicates that normoxic tissue has a PO₂ that is well above the PO₂ that will saturate 50% Mb. Given the signal to noise of the deoxyMb signal, a resting state cellular PO₂ that will desaturate Mb by ~10% will reveal a detectable proximal histidyl N₆H signal. None is observed.

During apnea, the MbO₂ clearly desaturates and reflects both a contribution to oxidative metabolism and a decrease in cellular PO₂. The latter should enhance the blood-to-muscle PO₂ gradient during apnea and promote O₂ transport into the cell (3, 31), provided arterial PO₂ and muscle blood flow are adequate throughout apnea. Although the relative contributions of Mb O₂ and blood-borne O₂ during sleep apnea are unknown at this time, O₂ flux to the mitochondria is apparently maintained during apnea. Such an interpretation is consistent with the constant high-energy phosphate levels observed in the 31P spectra. The chemical shift of the P₃ peak remains constant and does not indicate any pH change, consistent with the lack of postapneic lactate washout. Overall, the O₂ reservoir of Mb, the physiological alteration in blood flow, the enhanced capillary-to-cellular O₂ gradient, and any downregulation of metabolic activity serve to maintain oxidative phosphorylation without shifting to anaerobic glycolysis during the eupnea-to-apnea transition (5).

Conclusions

The solution studies have established the spectral features of M. angustirostris Mb and Hb. Both Mb and Hb are predominantly in one major isofrom. No conformational heterogeneity is detectable. During eupnea, the tissue PO₂ is sufficient to saturate Mb. During apnea, however, tissue PO₂ falls as reflected in the appearance of the deoxyMb signal. The study establishes the methodological basis for investigating metabolic responses during sleep apnea in M. angustirostris, which in turn provides insight into the regulation of O₂ metabolism during diving.

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