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RESEARCH ARTICLE

Functional properties of myoglobins from five whale species with different diving capacities

Signe Helbo* and Angela Fago

Department of Bioscience, Aarhus University, 8000 Aarhus C, Denmark

*Author for correspondence (signe.helbo@biology.au.dk)

SUMMARY

Whales show an exceptionally wide range of diving capabilities and many express high amounts of the O_2 carrier protein myoglobin (Mb) in their muscle tissues, which increases their aerobic diving capacity. Although previous studies have mainly focused on the muscle Mb concentration and O_2 carrying capacity as markers of diving behavior in whales, it still remains unexplored whether whale Mbs differ in their O_2 affinities and nitrite reductase and peroxidase enzymatic activities, all functions that could contribute to differences in diving capacities. In this study, we have measured the functional properties of purified Mbs from five toothed whales and two baleen whales and have examined their correlation with average dive duration. Results showed that some variation in functional properties exists among whale Mbs, with toothed whale Mbs having higher O_2 affinities and nitrite reductase activities (similar to those of horse Mb) compared with baleen whale Mbs. However, these differences did not correlate with average dive duration. Instead, a significant correlation was found between whale Mb concentration and average duration and depth of dives, and between O_2 affinity and nitrite reductase activity when including horse Mb. Despite the fact that the functional properties showed little species-specific differences *in vitro*, they may still contribute to enhancing diving capacity as a result of the increased muscle Mb concentration found in extreme divers. In conclusion, Mb concentration rather than specific functional reactivities may support whale diving performance.

Key words: oxygen binding, nitrite reductase, hydrogen peroxide.

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INTRODUCTION

In contrast to most terrestrial mammals with very limited breath-hold capabilities, diving marine mammals such as whales (cetaceans) are able to remain submerged for long periods of time. The most extreme divers are found within the toothed whales (odontocetes), with the sperm whale being able to dive to depths of more than 2000 m and stay submerged for more than 2 h (reviewed by Ponganis, 2011). Key adaptations that make these animals capable of such prolonged dives include: (1) an increased O_2 storage capacity in blood and muscle that makes it possible to rely largely on aerobic metabolism during normal diving (i.e. within the aerobic dive limit, defined as the dive duration beyond which lactate is produced) (Butler and Jones, 1997; Kooyman and Ponganis, 1998) and (2) a greater defense against oxidative stress as a result of increased mitochondrial production of reactive oxygen species (ROS) at reoxygenation (Cantú-Medellín et al., 2011; Bickler and Buck, 2007). However, knowledge on the molecular mechanisms basic to the diving physiology of these elusive animals is still limited because of the difficulty of obtaining biological samples.

Diving mammals typically have a high concentration of the O_2 carrier myoglobin (Mb) in their skeletal muscle [~10–95 mg g^{-1} (Ponganis, 2011)] compared with non-diving mammals [~3–10 mg g^{-1} (Snyder, 1983; Kayar et al., 1988)], an adaptation that allows them to maintain a prolonged aerobic metabolic activity of swimming muscle during dives by increasing O_2 reserves (Scholander, 1940; Butler and Jones, 1997) and facilitating O_2 diffusion within myocytes (Ponganis et al., 2008). In toothed whales, but not in baleen whales (mysticetes), the Mb concentration in muscle has been shown to be positively and significantly correlated with maximum dive duration and with body size (Noren and Williams, 2000). However, for some extreme whale divers, the beaked whales, the duration of a deep foraging dive can be approximately twofold longer than the estimated total O_2 carrying capacity, even when corrected for the lower metabolic rate owing to increased animal size (Tyack et al., 2006). This suggests that these whales may be able to decrease their O_2 consumption rate, which would prolong O_2 reserves and the aerobic dive limit even further during diving. Additionally, whales in general are also expected to possess an enhanced protection against the generation of ROS that may occur upon resurfacing/reoxygenation, especially after long dives, when tissue O_2 reserves have been depleted. As described below, the progressive formation of deoxygenated Mb in the muscle because of the release of O_2 during diving and the reoxygenation of Mb at resurfacing may both play a central adaptive role in such metabolic and protective mechanisms.

Besides O_2 storage and diffusion (Gros et al., 2010; Wittenberg and Wittenberg, 2003; Dasmeh and Kepp, 2012), other functions of Mb, associated with the protection against damaging effects of hypoxia and reoxygenation, have been discovered in recent years (Shiva et al., 2007; Hendgen-Cotta et al., 2008; Flögel et al., 2004). Specifically, deoxygenated Mb is able to reduce nitrite, an endogenous product of nitric oxide (NO) metabolism, to NO (Shiva et al., 2007). The NO generated downregulates mitochondrial respiration and reduces O_2 consumption during hypoxia (Shiva et al., 2007) by reversible inhibition of cytochrome *c* oxidase (Brown and Cooper, 1994). The Mb-mediated reduction

of nitrite to NO also protects against ischemia/reperfusion injury, as shown in the heart (Hendgen-Cotta et al., 2008). In hemoglobins (Hbs), this nitrite reductase activity has been shown to be positively correlated with O_2 affinity (Huang et al., 2005a), a correlation that may be of importance in the adaptive tolerance to hypoxia given that high-O_2-affinity Hbs are typical of hypoxia-tolerant animals (Huang et al., 2005a; Jensen, 2009). It is not known, however, whether for Mbs a high O_2 affinity correlates with hypoxia tolerance and/or with a high nitrite reductase activity. Additionally, Mb can act as a peroxidase and scavenge hydrogen peroxide (H_2O_2) (Yusa and Shikama, 1987; Flögel et al., 2004; Helbo et al., 2012), a major ROS produced endogenously when tissues are reoxygenated after hypoxia, thereby protecting against oxidative damage (Bickler and Buck, 2007; Flögel et al., 2004), as it would occur when whales reemerge from diving. Taken together, these enzymatic properties of Mb could contribute to the extreme tolerance to internal hypoxia displayed by whales during diving. In addition, although Mb concentration is known to be correlated with maximal dive durations (Noren and Williams, 2000), it is not known whether Mbs from long- and short-duration divers differ in any of these functional properties, which would indicate fine-tuning of Mb function according to dive performance.

In this study, we investigated whether Mb may contribute to whale dive performance by having different functional properties (O_2 affinities and kinetics, nitrite reductase and peroxidase activities) and/or by having different muscle concentrations. To this end, we purified and characterized Mbs from five cetacean species (three toothed whales and two baleen whales) that are known to differ in their diving performance. We measured values for O_2 affinities and kinetics, nitrite reductase and peroxidase activities of the purified Mbs from these species and tested for their correlation with the average dive duration known for the individual whale species. Additionally, we tested whether Mb enzymatic reactivities (nitrite reductase and peroxidase activities) and O_2 affinity are correlated in the mammalian Mbs (whales and horse) included in this study. Furthermore, we measured muscle Mb concentration (for minke whale and humpback whale) and examined whether this is correlated with average dive duration and dive depth in a range of whale species.

MATERIALS AND METHODS

A muscle sample from northern bottlenose whale (BNW) [*Hyperoodon ampullatus* (Forster 1770)] was obtained from a frozen specimen stranded in the Faroe Islands in 2010. Humpback whale (HW) [*Megaptera novaeangliae* (Borowski, 1781)] and arctic minke whale (MW) (*Balaenoptera acutorostrata* Lacépède 1804) muscle samples from one specimen each were obtained from whales caught by hunters in Nuuk, Greenland (CITES permission 11GL0806453 and 11GL0806452, respectively). Harbor porpoise (HP) [*Phocoena phocoena* (Linnaeus 1758)] muscle was obtained from one animal euthanized at the Dolfinarium Harderwijk, Holland. All muscle samples were frozen as fast as possible after being cut out of the whales and were kept at –80°C until purification of the Mbs. A frozen (–80°C) sample of purified sperm whale (SW) (*Physeter macrocephalus* Linnaeus 1758) Mb obtained from a dead specimen stranded at Rømø, Denmark, in 1996 was kindly provided by Prof. Roy E. Weber (Aarhus University). For functional comparisons, purified horse heart Mb (Sigma-Aldrich, St Louis, MO, USA) was used as representative of Mb from a non-diving mammal. Chemicals were from Sigma-Aldrich unless otherwise stated and water was Milli-Q grade.

Purification of myoglobins

Mb from BNW, HW, MW and HP was purified from muscle as previously described (Helbo and Fago, 2011). In brief, Mb was precipitated from muscle homogenates by ammonium sulphate fractionation (40 and 100%) followed by fast protein liquid chromatography gel filtration using a Tricorn Superdex 75 10/300 GL column (GE Healthcare, Broendby, Denmark) equilibrated with 50 mmol l^{-1} Tris, 0.5 mmol l^{-1} EDTA, 3 mmol l^{-1} dithiothreitol (DTT), 0.15 mol l^{-1} NaCl, pH 8.3 to separate Mb from contaminating Hb. Horse Mb was converted from the ferric (met) to the ferrous (oxy) form by standard procedures after adding solid dithionite and desalting on a PD10 column (GE Healthcare) equilibrated with the gel filtration buffer described above. Mb purity was assessed by sodium dodecyl sulfate (SDS) and isoelectric focusing (IEF) on polyacrylamide gels (Phast System, GE Healthcare). Isoelectric points (pIs) of the native purified Mbs were obtained by IEF in the pH 3–9 range, using a Broad pI Kit (pH 3–10, GE Healthcare). The heme oxygenation/oxidation state was assessed by ultraviolet–visible (UV-vis) absorption spectroscopy in the range 400–700 nm by using absorption peaks known for Mb (Antonini and Brunori, 1971). All whale Mbs were purified as oxy derivatives, without detectable met heme. This shows that all whale Mbs were in good condition despite muscle samples and purified Mbs being of varying age.

Myoglobin concentration

Muscle Mb concentrations for the toothed whales HP, BNW and SW, and for horse were obtained from previously published values (Noren and Williams, 2000; Scholander, 1940; Kayar et al., 1988). The muscle concentrations of the baleen whale (HW and MW) Mbs were measured spectrophotometrically using a modified version of the method by Reynafarje, which takes advantage of characteristic spectral differences to differentiate between Mb and Hb in contaminating blood (Reynafarje, 1963). In this procedure, thawed muscle samples (~0.2 g) were homogenized on ice using an Ultra-Turrax T25 homogenizer (IKA Labortechnik, Staufen, Germany) in 40 mmol l^{-1} potassium phosphate buffer (pH 6.6 for HW, and pH 7.7, 3 mmol l^{-1} DTT, 0.5 mmol l^{-1} EDTA for MW) at a buffer to tissue ratio of 19.25 ml buffer g^{-1} wet tissue (Reynafarje, 1963). Samples were centrifuged for 50 min at 15,000 ***g*** at 4°C, and the supernatant was equilibrated with pure CO gas for 3 min. Dithionite (~0.001 g) was added and the sample was equilibrated with CO for an additional 1 min. Absorbance was recorded at 538 and 568 nm using an HP 8543 UV-vis diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA, USA) and the concentration of Mb [mg g^{-1} wet mass (WM)] was calculated using the formula: $[Mb]=(A_{538}–A_{568})\times 117.3$, where the difference in absorbance (A) at 538 and 568 nm is multiplied by a constant containing the molar extinction coefficients of carbonyl Hb and Mb, the molecular weights of Hb and Mb and the volume of buffer to mass of tissue (Reynafarje, 1963). No changes in absorbance were found after longer CO equilibration.

Oxygen equilibria

O_2 binding curves were determined using a modified diffusion chamber technique previously described (Sick and Gersonde, 1969; Weber, 1992; Weber et al., 2000). Briefly, water-saturated gas mixtures of O_2 or air and ultrapure (>99.998%) N_2 created by Wösthoff gas mixing pumps (Wöstoff, Bochum, Germany) were used to equilibrate a thin smear (4 µl, ~100 µmol l^{-1} heme) of Mb solution with stepwise increases in oxygen tension (P_{O_2}). Changes in absorbance upon oxygenation were recorded continuously at 436 nm by a photomultiplier (model RCA 931-A, Hamamatzu,

Hamamatzu City, Japan) and an Eppendorf model 1100M photometer (Hamburg, Germany). The absorbance signal was digitalized and measured using a computer with the in-house custom-made data acquisition software Spectrosampler. P_{50} (P_{O_2} at half-saturation) and n_{50} (cooperativity) values were calculated from the zero intercept and slope of Hill plots, respectively: $\log(Y)/(1-Y)$ *versus* $\log P_{O_2}$, where Y is the fractional saturation of Mb. Each curve consists of four to five saturation steps. Experiments were carried out in duplicate in 50 mmol l^{-1} Tris, 0.5 mmol l^{-1} EDTA, 3 mmol l^{-1} DTT, 0.15 mol l^{-1} NaCl, pH 8.3 at 37°C.

O_2 dissociation kinetics

O_2 dissociation rates (k_{off}; s^{-1}) were measured using an OLIS RSM 1000 UV/Vis rapid-scanning stopped-flow spectrophotometer (OLIS, Bogart, GA, USA) coupled to a Dell computer with OLIS data collection software. In the stopped flow, oxygenated Mb (~10 µmol l^{-1} heme in 200 mmol l^{-1} Tris, pH 7.4) was mixed at a 1:1 ratio with 40 mmol l^{-1} dithionite prepared in deoxygenated buffer (200 mmol l^{-1} Tris, pH 7.4) and spectra (~390–600 nm, 1000 s^{-1}) were collected over time (~0.2 s). k_{off} rates were calculated from the monoexponential changes of the absorbance spectra using OLIS multiple wavelength data analysis software (SVD). The O_2 equilibrium constant (K; µmol l^{-1}) was calculated by multiplying the measured P_{50} values by the solubility of O_2 in water at 37°C (1.407 l mmol^{-1} Torr^{-1}) (1 Torr≈133 Pa) (Boutilier et al., 1984). O_2 association rates (k_{on}; l µmol^{-1} s^{-1}) were derived from the relationship $k_{on}=k_{off}/K$, assuming that the P_{50} of Mbs is independent of pH. Rates were measured at 25 and 37°C.

Nitrite reduction

The reaction of nitrite with the Mbs (~10 µmol l^{-1} heme in deoxygenated 200 mmol l^{-1} Tris, 0.5 mmol l^{-1} EDTA 0.1 mol l^{-1} KCl, pH 7.4) was measured under pseudo-first-order conditions in the presence of dithionite (~200–300 µmol l^{-1}) and under anaerobic conditions as previously described (Shiva et al., 2007; Pedersen et al., 2010; Helbo et al., 2012). The nitrite concentration of freshly made stock solutions (~20 mmol l^{-1}) was verified by the Griess reaction (Giustarini et al., 2008) prior to experiments. Varying amounts of nitrite was added anaerobically to the Mb solution *via* a Hamilton gastight syringe (Bonaduz, Switzerland) and spectral changes were measured over time in the range 390–650 nm using an HP 8543 UV-vis diode array spectrophotometer. At the end of the reaction, deoxy Mb (peak at 555 nm) was fully converted to the Fe-NO form (peaks at 545 and 572 nm) as predicted by the reaction stoichiometry (Shiva et al., 2007; Pedersen et al., 2010; Helbo et al., 2012). Observed rates (k_{obs}; s^{-1}) were obtained by fitting absorbance traces at 555 nm to single exponential decay functions. The pH of the reaction mixture was checked after each experiment. Second-order rate constants (l mol^{-1} s^{-1}) were obtained from the slopes of linear plots of observed rates as a function of nitrite concentrations. Experiments were performed at 25°C and at pH 7.4 to allow for comparison with rate constants determined in previous studies.

Reaction with hydrogen peroxide (peroxidase activity)

To investigate the role of whale Mbs in the overall protection against ROS, H_2O_2 (~1 mmol l^{-1} in water) was added to oxygenated Mb solutions (~10 µmol l^{-1} heme, 20 mmol l^{-1} Tris pH 7.5) at a 2:1 H_2O_2/heme molar ratio, as previously described (Helbo et al., 2012). The changes in absorbance spectra were in agreement with those of carp Mbs (Helbo et al., 2012) and with the reaction scheme described for the reaction between oxy Mb and H_2O_2 (Yusa and Shikama, 1987). The decrease in absorbance at 416 nm over time was monitored at 25°C using an HP 8543 UV-vis diode array spectrophotometer. To correct for autoxidation of the Mbs, absorbance of samples with water added instead of H_2O_2 was measured in parallel over time. Experiments were performed at 25°C to allow for comparisons with other studies. Observed rates were obtained by fitting a single exponential decay function to the absorbance measured at 420 nm. Rates were measured in duplicate.

Dive duration

Functional properties were correlated to average dive duration values of long, foraging dives reported for the five whale species (Table 1). To investigate whether Mb concentration was dependent on whale dive performance, Mb concentration from the investigated species and from additional whale species was correlated with available data on average dive duration and on average dive depth. Species, values and references are listed in Table 1.

Table 1. Myoglobin concentration, average dive duration and average dive depth for selected whales

	[Mb] (mg g^{-1} wet mass)	Average dive duration (min)	Average dive depth (m)	Reference
Toothed whales				
Bottlenose whale	63	37	1060	Scholander, 1940; Hooker and Baird, 1999
Sperm whale	54	45	666	Scholander, 1940; Watwood et al., 2006
Cuvier's beaked whale	43	58	1070	Noren and Williams, 2000; Tyack et al., 2006
Beluga whale	34	13	n.d.	Noren and Williams, 2000; Martin and Smith, 1999
Spotted dolphin	25	1.5	23	Castellini and Somero, 1981; Scott and Chivers, 2009
Bottlenose dolphin	27	0.5	20	Noren and Williams, 2000; Mate et al., 1995; Ponganis, 2011
Harbor porpoise	40	1	25	Noren and Williams, 2000; Westgate et al., 1995
Baleen whales				
Bowhead whale	35	10.5	67	Noren and Williams, 2000; Krutzikowsky and Mate, 2000
Fin whale	24	6.5	98	Noren and Williams, 2000; Croll et al., 2001
Humpback whale	16	8	143	Present study; Goldbogen et al., 2008
Minke whale	22[a]	4.5	n.d.	Present study, and Scholander, 1940; Stern, 1992
Horse	10	n.d.	n.d.	Kayar et al., 1988

Average dive duration is the duration of feeding dives (long, deep dives).

[Mb] references are given first, then references on average dive duration, followed by references on average dive depth (if different from that on average dive duration).

[a]Value is the average of the concentration measured in this study (7 mg g^{-1}) and the concentration calculated from Scholander, 1940 by Dolar et al., 1999 (37 mg g^{-1}).

n.d., not determined.

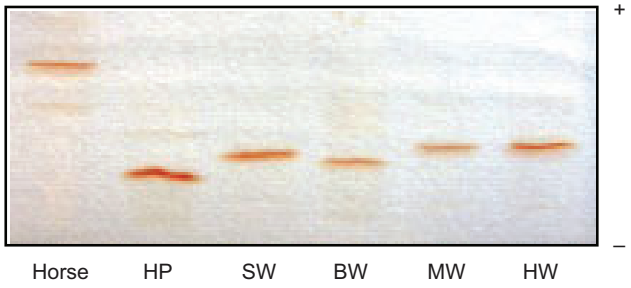

Fig. 1. Unstained isoelectric focusing polyacrylamide gel (pH 3–9) of purified whale [harbor porpoise (HP), sperm whale (SW), bottlenose whale (BNW), minke whale (MW), humpback whale (HW)] and horse myoglobins (Mbs).

Statistics

Least-square linear regression analyses were used to test for correlations of O_2 affinity, nitrite reductase and peroxidase activities and Mb concentration with average dive duration, of Mb concentration with average dive depth, and of O_2 affinity with peroxidase and nitrite reductase activities. Results were taken as significant when $P \leq 0.05$. Values are presented as means ± s.d. when $N \geq 3$. Statistical tests were performed using SigmaPlot 11 (Systat Software, San Jose, CA, USA).

RESULTS

Whole Mbs were successfully purified from skeletal muscles as judged by SDS-PAGE and from ratios of Soret (~416 nm) to protein peak (260 nm) absorbance >5 (data not shown). An unstained IEF gel of the purified Mb samples (Fig. 1) showed only one red band, which indicates that whale muscles contain one Mb isoform, having higher pI values (SW, 7.89; BNW, 7.91; HW, 7.77; MW, 7.78; HP, 8.00) than horse Mb (7.36).

The concentrations of Mb in muscle tissues from the baleen whales (HW and MW) were 15.9±0.8 mg g^{-1} WM (N=8, from one individual) and 7.3±1.0 mg g^{-1} WM (N=4, from one individual), respectively. However, the value for the MW Mb concentration may be a low estimate because of some precipitation of the homogenized sample. In Table 1, showing the data for Mb concentration, we therefore report the average of our value for MW Mb and a value (37 mg g^{-1} WM) calculated from the measured muscle O_2 capacity (Scholander, 1940; Dolar et al., 1999). Additional published whale Mb muscle concentrations are also listed in Table 1, together with available data on average dive duration and on average dive depth. When plotting Mb concentration as a function of average dive duration (Fig. 2A) and dive depth (Fig. 2B), the regression analysis shows that Mb concentration is positively and significantly correlated with both (r^2=0.522, P=0.012, N=11; r^2=0.610, P=0.013, N=9, respectively). As data on average dive depth are not available for the minke whale, only average dive duration was used to investigate further the correlations with enzymatic reactivities of the purified Mbs.

Whale Mb O_2 binding curves were hyperbolic (Fig. 3) and P_{50} values for the toothed whale (BNW, SW and HP) Mbs were similar to that for horse Mb (Table 2). In contrast, baleen whale (HW and MW) Mbs had slightly higher P_{50} values and thus lower O_2 affinities (Table 2). Mbs from all species showed cooperativity values (n_{50}) close to 1 (range: 0.9–1.1; Fig. 3), which is consistent with the monomeric structure of the Mbs and further confirms that Hb was not present as a contaminant.

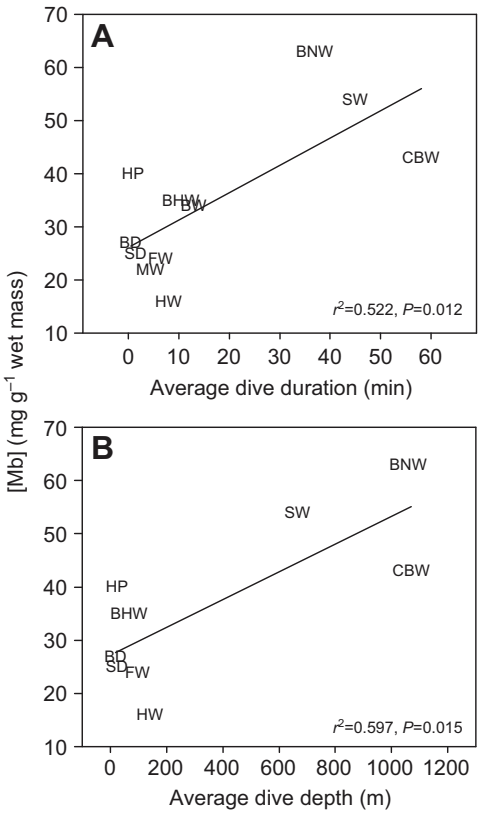

Fig. 2. Mb concentration as a function of (A) average dive duration and (B) average dive depth of whales. Species abbreviations are: harbor porpoise (HP), sperm whale (SW), bottlenose whale (BNW), minke whale (MW), humpback whale (HW), Cuvier's beaked whale (CBW), fin whale (FW), bowhead whale (BHW), bottlenose dolphin (BD), spotted dolphin (SD). Data from this study are the Mb concentrations of humpback whale and minke whale. Values and references are reported in Table 1. Least-square linear regressions and levels of significance (r^2 and P values) are indicated.

O_2 dissociation stopped-flow kinetics showed that the low-O_2-affinity baleen whale Mbs had slightly higher k_{off} rates and slightly lower k_{on} rates than Mbs from toothed whales. Measured (k_{off}) and calculated (k_{on}) values of Mb O_2 kinetics are listed in Table 2.

Apparent second-order rate constants for the reduction of nitrite to NO by whale and horse deoxy Mbs (at 25°C) were in the range of 3–6.6 l mol^{-1} s^{-1} (Fig. 4). These results show that deoxy Mbs of toothed whales [HP, 6.5±0.2 l mol^{-1} s^{-1}; BNW, 6.6±0.3 l mol^{-1} s^{-1}] are more than twofold faster than those of baleen whales [HW, 3.0±0.1 l mol^{-1} s^{-1}; MW, 2.6±0.2 l mol^{-1} s^{-1}] at reducing nitrite to NO. The nitrite reductase rates of horse [5.4±0.4 l mol^{-1} s^{-1}] and SW [4.1±0.4 l mol^{-1} s^{-1}] Mbs are intermediate.

When reacted with a twofold molar excess of H_2O_2, the whale Mbs investigated here showed similar rates (BNW, 0.51 s^{-1}; SW, 0.56 s^{-1}; HW, 0.55 s^{-1}; MW, 0.41 s^{-1}; HP, 0.39 s^{-1}), whereas horse Mb was slightly faster (0.70 s^{-1}) at removing H_2O_2.

Mb concentrations, O_2 affinities, nitrite reductase activities and peroxidase activities are plotted as a function of the average dive duration of the whale species in Fig. 5. The regression analyses show that the correlations are not significant (Mb concentration: r^2=0.638, P=0.105, N=5; O_2 affinity: r^2=0.405, P=0.248, N=5; nitrite reductase activity: r^2=0.051, P=0.716, N=5; peroxidase activity: r^2=0.487, P=0.190, N=5). Conversely, the functional properties – nitrite reductase activity and P_{50} – were significantly correlated (r^2=0.719,

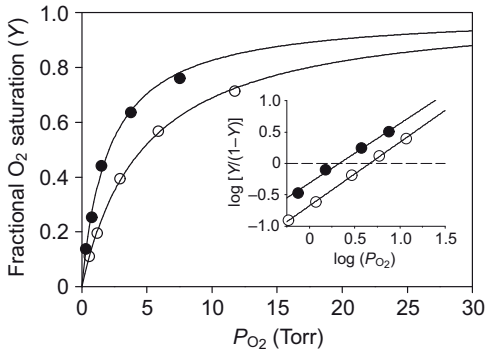

Fig. 3. Oxygen binding equilibria and kinetics of whale Mbs. Representative O_2 binding curves for minke whale (open symbols) and bottlenose whale (closed symbols) Mbs measured in 50 mmol l^{-1} Tris, 0.5 mmol l^{-1} EDTA, 3 mmol l^{-1} DTT, 0.15 mol l^{-1} NaCl, pH 8.3 at 37°C. Hyperbolic fitting of the data is indicated. Inset shows Hill plots of the same data with the dotted line indicating 50% saturation.

Table 2. Mb O_2 affinity equilibrium constants (P_{50} and K), and dissociation (k_{off}) and association (k_{on}) rates

Species	P_{50} (Torr)	K (μmol l^{-1})	k_{off} (s^{-1})	k_{on} (l μmol s^{-1})
Bottlenose whale	2.2	3.1	79.0 (19.7)	25.5
Sperm whale	2.7	3.8	88.2 (20.1)	23.2
Harbor porpoise	2.7	3.8	88.0 (21.5)	23.2
Humpback whale	3.9	5.5	97.4 (27.7)	17.8
Minke whale	4.6	6.5	92.7 (25.0)	14.3
Horse	2.1	3.0	85.8 (20.9)	29.0

Values were measured at 37°C (values at 25°C in parentheses). N=3–5 in all experiments.

P=0.033, N=6; Fig. 6A), meaning that a high nitrite reductase activity is associated with a high O_2 affinity (i.e. a lower P_{50}) in the mammalian Mbs (including horse) of this study. No significant correlation was found between peroxidase activity and P_{50} (r^2=0.244, P=0.319, N=6; Fig. 6B).

DISCUSSION

Earlier studies have mainly focused on Mb concentration and on muscle O_2 carrying capacity as markers of diving behavior in marine mammals, while it has remained unexplored whether Mbs from whales with different diving capacities also differ in their functions as O_2 carriers and in their nitrite reductase and peroxidase enzyme activities. These are all properties that are expected to be of importance in matching O_2 supply with consumption during diving and in limiting oxidative stress after diving. As discussed below, even though these properties show little species-specific difference *in vitro* for the isolated Mbs, they may be amplified *in vivo* as a result of the massive increase in muscle Mb concentration present in extreme divers.

Mbs from the three toothed whales included in this study (SW, BNW and HP) are similar to horse Mb in having relatively high O_2 affinities and high nitrite reductase activities (Fig. 5A,B). The P_{50} values are all below 3 Torr at 37°C, which is typical for mammalian Mbs (Antonini and Brunori, 1971; Nichols and Weber, 1989). Also, the nitrite reductase activities of toothed whale and horse Mbs (4.1–6.61 mol^{-1} s^{-1}) are similar to reported values for sperm whale [5.61 mol^{-1} s^{-1} (Tiso et al., 2011)] and horse Mb [61 mol^{-1} s^{-1} (Huang et al., 2005b)], although one study on horse Mb reported a lower reactivity [2.91 mol^{-1} s^{-1} (Tiso et al., 2011)]. Compared with toothed whales and other mammals, baleen whales (MW, HW) have Mbs with slightly lower O_2 affinities and lower nitrite reductase rates (Fig. 5A,B). The low O_2 affinities of baleen whale Mbs are explained by slight increases and decreases in O_2 dissociation and association rates, respectively. Together with the similar peroxidase activity found for whale Mbs (Fig. 5C), our data show that these minor differences in Mb reactivities are not sufficient to contribute significantly to the average dive duration. Instead, genetically determined functional properties of the Mbs appear similar overall in phylogenetically related whales, such as HP, SW and BNW, that differ widely in diving behavior and capability. The data also demonstrate that a correlation exists between the high O_2 affinity (low P_{50}) and the nitrite reductase activity of mammalian Mbs (Fig. 6), as intrinsic specific properties of the Mbs. Such correlation may be due to a lower reducing potential of the heme, as proposed for Hbs (Huang et al., 2005a), or to a more accessible heme pocket, which would suggest some common molecular mechanism in the regulation of heme reactivity towards O_2 or nitrite in Mbs. In contrast, the peroxidase activity does not correlate with either average diving duration or O_2 affinity in mammalian Mbs, at least not under the conditions investigated here. This finding suggests that in mammals, other enzymes with a more specific peroxidase and catalase activity than Mb may be of greater importance in the detoxification against H_2O_2 and other ROS.

In general, the correlation between Mb O_2 affinity and nitrite reductase activity may be adaptive in hypoxia-tolerant animals; it may (1) improve facilitated diffusion at low P_{O_2} gradients at the blood–muscle interface and (2) increase the rate of NO generated from nitrite per unit time as Mb becomes progressively deoxygenated. In whales, an increased production of NO could be beneficial during long dives by slowing down mitochondrial respiration, which would prolong O_2 reserves (Shiva et al., 2007) and increase cellular protection against the damaging effects of reoxygenation after hypoxic episodes (Hendgen-Cotta et al., 2008). Although this could be of particular importance in extreme divers such as SW and BNW, these intrinsic functional properties of the Mbs alone cannot account for the Mb contribution to average dive duration unless Mb concentration is taken into account, as explained below.

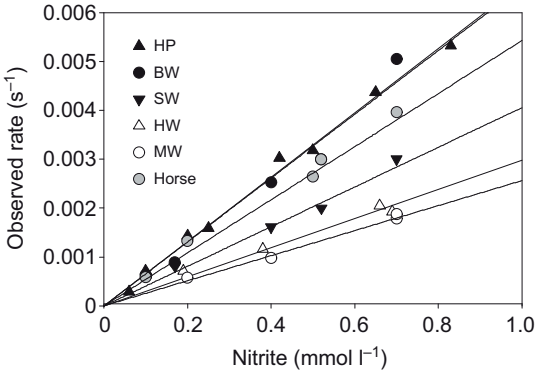

Fig. 4. Nitrite reductase activity of Mbs from toothed whales (closed symbols) and baleen whales (open symbols) compared with horse Mb (grey symbols). Observed rates of the reaction of deoxy Mbs with nitrite (conditions: 200 mmol l^{-1} Tris, 0.5 mmol l^{-1} EDTA, 0.1 mol l^{-1} KCl, pH 7.4 at 25°C) are plotted as a function of nitrite concentration. Second-order rate constants for the nitrite reductase activities are given by the slopes of the linear regression of the data.

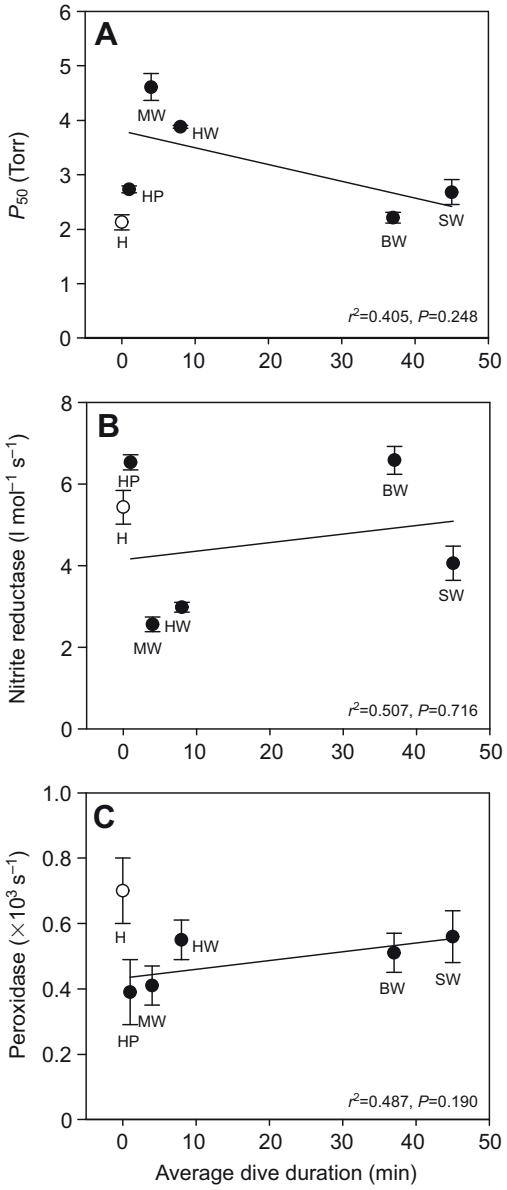

Fig. 5. (A) P_{50}, (B) nitrite reductase activity and (C) peroxidase activity as a function of average dive duration for toothed whales [bottlenose whale (BNW), sperm whale (SW) and harbor porpoise (HP)] and baleen whales [humpback whale (HW) and minke whale (MW)]. Least-square linear regressions and levels of significance (r^2 and P values) are indicated. Horse (H, white circle) is shown as an example of a non-diving mammal, but is not included in the regression analyses. Values are means ± s.d.

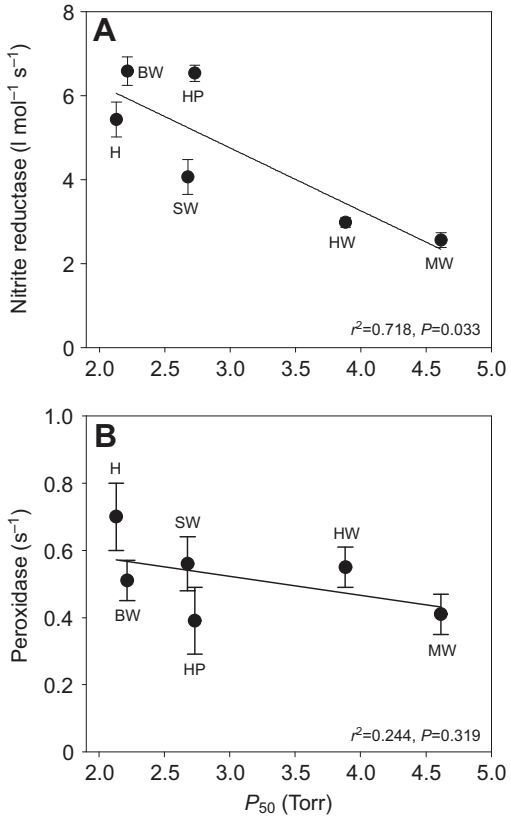

Fig. 6. P_{50} as a function of nitrite reductase activity for bottlenose whale (BNW), sperm whale (SW), harbor porpoise (HP), humpback whale (HW), minke whale (MW) and horse (H). Linear regression analyses and levels of significance (r^2 and P values) are indicated. Values are means ± s.d.

When including literature data for additional whale species, we found a significant and positive correlation between average dive duration and Mb concentration for whales, with toothed whales showing the highest durations and the highest Mb concentrations (Fig. 2, Table 1). This extends the results of a previous study where maximum dive duration (which may far exceed the aerobic dive limit) was found to be correlated with Mb concentration in toothed whales but not in baleen whales (Noren and Williams, 2000).

The Mb concentrations of some of the whale species differ from what would be predicted from their average dive durations, and in particular the harbor porpoise has a very high Mb concentration despite a modest dive performance. Several reasons may underlie these deviations. First, body size influences the specific metabolism and it may therefore affect O_2 demand and the relationship between dive performance and Mb concentration. This may explain at least in part why the small harbor porpoise expresses comparably high amounts of Mb. Second, whales show great differences in behavior and foraging strategies, with the toothed whales spending more energy on capturing prey than the filter-feeding baleen whales. This, in turn, may also affect O_2 consumption and thus the need for Mb. Data shown in Fig. 2 suggest that the Mb concentration tends to be higher in toothed whales than in baleen whales showing the same diving abilities. Future investigations on other whale species, especially those with intermediate diving abilities, and on variations in Mb concentration among individuals of the same species, studies that were not feasible here, would represent a useful addition to this study.

Besides increasing the storage capacity for O_2 in muscle and O_2 supplied to the mitochondria per unit time (Ponganis et al., 2008), a higher concentration of Mb will also effectively increase the concentration of enzyme available and thus the rate of nitrite reduction and hydrogen peroxide removal of the muscle tissue. Thus, despite the lack of correlation between the species-specific functional properties of whale Mbs and the average dive duration, simply because of a higher protein concentration, all Mb functions will be enhanced *in vivo* in long-duration divers compared with short-duration divers. As a case in point, for a given nitrite concentration, the nitrite reductase activity coupled with the high Mb concentration would increase NO production per unit time in the skeletal muscle

of BNW approximately eightfold compared with HW and horse muscle when O_2 reserves have been depleted. Furthermore, a recent study shows that plasma nitrite levels are elevated in harbor porpoise in comparison with other mammals (Soegaard et al., 2012), indicating that relatively high nitrite levels may also be present in whale skeletal muscle.

In conclusion, we found that some variation exists among whale Mbs in O_2 binding, nitrite reductase activity and rate of reaction with H_2O_2, but this variation is not correlated with average dive duration. Instead, the variation may reflect phylogeny and differences in foraging strategies, as the greatest differences in Mb functional properties were found between the toothed and baleen whale suborders, with toothed whales having comparably higher O_2 affinities and higher nitrite reductase activities. Furthermore, an increased Mb O_2 affinity is correlated with an increased nitrite reductase activity in mammals in general, which may be adaptive in hypoxia-tolerant animals. Average dive duration and depth significantly correlate with Mb muscle concentration in whales, and we conclude that such correlation will entail a consequent enhancement of Mb functions *in vivo*, including capability for O_2 storage and for nitrite reduction to NO, which may considerably prolong dive performance in extreme divers. Therefore, it appears that differences in Mb concentration rather than differences in specific functional reactivities may contribute to dive performance in whales.

LIST OF ABBREVIATIONS

BNW	bottlenose whale
CO	carbon monoxide
H_2O_2	hydrogen peroxide
Hb	hemoglobin
HP	harbor porpoise
HW	humpback whale
K	equilibrium constant
k_{off}	O_2 dissociation rate
k_{on}	O_2 association rate
Mb	myoglobin
MW	minke whale
n_{50}	cooperativity
NO	nitric oxide
P_{50}	P_{O_2} at half-saturation
pI	isoelectric point
P_{O_2}	O_2 tension
ROS	reactive oxygen species
SW	sperm whale
WM	wet mass

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