Functional differentiation of myoglobin isoforms in hypoxia-tolerant carp indicates tissue-specific protective roles

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Helbo S, Dewilde S, Williams DR, Berghmans H, Berenbrink M, Cossins AR, Fago A. Functional differentiation of myoglobin isoforms in hypoxia-tolerant carp indicates tissue-specific protective roles. Am J Physiol Regul Integr Comp Physiol 302: R693–R701, 2012. First published December 14, 2011; doi:10.1152/ajpregu.00501.2011. — Because of a recent whole genome duplication, the hypoxia-tolerant carp and goldfish are the only vertebrates known to possess two myoglobin (Mb) paralogs. One of these, Mb1, occurs in oxidative muscle but also in several other tissues, including capillary endothelial cells, whereas the other, Mb2, is a unique isoform specific to brain neurons. To help understand the functional roles of these diverged isoforms in the tolerance to severe hypoxia in the carp, we have compared their O2 equilibria, carbon monoxide (CO) and O2 binding kinetics, thiol S-nitrosation, nitrite reductase activities, and peroxidase activities. Mb1 has O2 affinity and nitrite reductase activity comparable to most vertebrate muscle Mbs, consistent with established roles for Mbs in O2 storage/delivery and in maintaining nitric oxide (NO) homeostasis during hypoxia. Both Mb1 and Mb2 can be S-nitrosated to similar extent, but without oxygenation-linked allosteric control. When compared with Mb1, Mb2 displays faster O2 and CO kinetics, a lower O2 affinity, and is slower at converting nitrite into NO. Mb2 is therefore unlikely to be primarily involved in either O2 supply to mitochondria or the generation of NO from nitrite during hypoxia. However, Mb2 proved to be significantly faster at eliminating H2O2, a major in vivo reactive oxygen species (ROS), suggesting that this diverged Mb isoform may have a specific protective role against H2O2 in the carp brain. This property might be of particular significance during reoxygenation following extended periods of hypoxia, when production of H2O2 and other ROS is highest.

nitric oxide; fish; oxygen binding; nitrite reductase; hydrogen peroxide

FOR DECADES myoglobin (Mb), the O2 carrier of skeletal and heart muscle of vertebrates, was believed to be expressed as a single isoform only in oxidative muscle tissue where it facilitates intracellular O2 diffusion and storage (17, 49). By using a postgenomic screening technique, Fraser et al. (13) discovered in 2006 that in the common carp (Cyprinus carpio) the muscle Mb (Mb1) is also expressed at low levels in a range of other tissues, including brain, liver, kidney, and gills. Surprisingly, a second Mb isoform (Mb2), expressed at low levels only in the brain, was also found in the common carp (13) and subsequently in the closely related goldfish (35). These are the only two vertebrate species known to express two separate Mb isoforms. These two isoforms diverged after a postulated recent whole genome duplication event ~12 million years ago (7), generating tetraploid genomes. As a result, many duplicate isoforms have become fixed, often with tissue-specific or response-specific patterns of expression (10).

The common carp and other members of the Cyprinidae are among the very few vertebrates that are able to tolerate periods of severe environmental hypoxia, both diurnally and for prolonged periods, with goldfish and crucian carp even surviving months of extreme hypoxia during winter in ice-covered ponds. This is accomplished in part by a strong metabolic depression to preserve energy while selectively maintaining basic functions, particularly in the brain (2, 30). Fixation of the unique brain-specific Mb2 isoform may be linked to this exceptional hypoxia tolerance. This idea is supported by the upregulation of Mb transcript and total Mb protein expression in the carp liver, gills, and brain following 5 days of hypoxia (6, 13) and by a twofold increase in goldfish Mb2 expression after 2 days exposure to hypoxia (35). In the carp brain, Mb1 has been located in capillary endothelial cells while Mb2 is found in neurons (6). This suggests that the two isoforms possess quite different functions, which may well account for some part of the hypoxia tolerance of the tetraploid Cyprinidae fish.

These discoveries on carp Mb expression have occurred against a backdrop of radically altered understanding of Mb functions other than O2 binding, notably including cellular protection against the effects of hypoxia and reoxygenation. Seminal studies using Mb-knockout mice demonstrated that the deoxygenated Mb formed during hypoxia can reduce nitrite to nitric oxide (NO) (40), which reversibly inhibits cytochrome c oxidase and lowers myocardial O2 consumption when O2 availability is limited (32). Furthermore, Mb may reduce the massive production of the reactive oxygen species (ROS) generated upon reoxygenation after prolonged hypoxia (2) by metabolizing a major endogenous ROS such as hydrogen peroxide (H2O2), thereby protecting cells from oxidative damage subsequent to hypoxia (12). Furthermore, cysteine (Cys) residues on Mb may become S-nitrosated (i.e., covalently bound to a NO moiety as SNO) (26, 33, 39), which may allosterically regulate heme oxygenation, as found in rainbow trout Mb (21), and be able to further contribute to NO homeostasis under hypoxia. Although well described in mammals, these novel functions of Mb have so far only been examined to very little extent in fish.

We here test the hypothesis that Mb1 and Mb2 from the common carp have different molecular functional properties that could contribute, at least in part, to the hypoxia tolerance of this species. To this end we have compared Mb1 and Mb2 O2 equilibrium curves and sensitivities to cofactors (pH, temperature, and lactate), O2 and carbon monoxide (CO) kinetics,
nitrite reductase activities, and peroxidase activities and have examined Cys reactivities and O₂ affinities of the S-nitrosated Mbs. Also, we have analyzed the molecular phylogeny of Mb1 and Mb2. This study is the first to compare the functional properties of two Mb isoforms expressed in the same organism. Our results indicate that carp Mb1 and Mb2 maintain different and tissue-specific functional roles and suggest a specific role of Mb2 in the protection of the carp brain during hypoxia and reoxygenation.

**MATERIALS AND METHODS**

Because of its low levels of expression in the brain, Mb2 was obtained as recombinant protein. Mb1 was obtained as either recombinant (rec-Mb1) or as native protein (Mb1) from heart tissue. Procedures are described below.

**Cloning, expression, and purification of Mb2 and rec-Mb1.** Full-length double-stranded cDNA clones of carp Mb1 (MYO-1) and Mb2 (MYO-2) were obtained by PCR amplification of cDNA synthesized from carp heart and brain RNA, respectively (first-strand cDNA synthesis-Superscript III, PCR-Platinum PfX; Invitrogen). PCR primers were designed to produce full-length coding regions of MYO-1 and MYO-2 incorporating a TAA stop codon. The primers were flanked by NdeI restriction sites at the 5’-end and XhoI restriction sites at the 3’-end. The primers also had additional flanking restriction sites of EcoRI (5’) and BamHI (3’) for optional subcloning and to improve digestion efficiency of NdeI and XhoI. PCR products for MYO-1 and MYO-2 were digested with NdeI and XhoI (New England Biolabs) and similarly the cloning vector pet21a (Novagen) was digested with the same restriction enzymes. Purified PCR product and Antartic phosphatase (NEB)-treated vector were then ligated overnight at 16°C using T4 DNA ligase (Invitrogen). An aliquot of the ligation mixture was used for electroporation into DH10B electropotentant cells (Invitrogen) and grown overnight on LB-ampicillin agar plates. The resulting clones were screened for the presence of MYO-1 and MYO-2 inserts by using a tip end of bacterial colony as a template for PCR. A selection of positive clones was grown on to produce bulk plasmid. The inserts of selected clones were sequenced twice (GATC procedures are described below).

**Purification of native Mb1 from carp heart.** Purification of native Mb1 from carp heart was done using the Superdex 75 column in 0.1 M HEPES buffer as a function of temperature (18, 20, 25, and 32°C), pH (6.4, 7.4, and 8.3), and in the absence and presence of lactate (10 and 100 mM at pH 6.5 for Mb1 and at pH 7.4 for Mb2). Briefly, in this method, a water-saturated gas mixture of O₂ or air and ultrapure (99.999%) nitrogen (N₂) created by Wösthoff (Bochum, Germany) gas mixing pumps was used to equilibrate a thin smear of Mb solution placed in a modified diffusion chamber equipped with a photomultiplier (model RCA 931-A) and an Eppendorf model 1100 M photometer coupled to a potentiometric linear recorder. In each experiment (∼30 min) the O₂ was increased in a stepwise fashion, and the sample was allowed to equilibrate while the absorption of Mb was continuously recorded at 436 nm to obtain the percent saturation at each O₂. For reference, 0 and 100% O₂ saturation levels were obtained by equilibrating with pure N₂ and O₂, respectively, at the beginning and end of each experiment. Experiments were done in duplicate or triplicate. P₅₀ (P₅₀ at half-saturation) and cooperativity (n₅₀) were determined from the zero intercept and slope, respectively, of the Hill plot: log₅₀[(1 − Y)] vs. logP₅₀, with Y as the fractional saturation. The overall heat of oxygenation (∆H) was calculated from the slope of the Van't Hoff plot: logP₅₀ vs. 1/T (50). A few curves were measured to confirm the equivalence between native, heart-purified and recombinant Mb1.

**O₂ kinetics.** To measure O₂ association (kₐ) and dissociation constants (kₐd) of Mb1 and Mb2, laser flash photolysis experiments were performed in different buffers equilibrated in different O₂-CO gas mixtures, using an Edinburg Instruments LP920 model (Livingson, UK). A frequency-doubled Q-switched Nd:YAG laser (Spectra Physics Quanta-ray, Newport, CA) at 532 nm was used. In these reactions, MbCO is prepared in 100 mM phosphate buffer containing 750 μM CO and 312.5 μM O₂, 500 μM CO and 625 μM O₂, and 250 μM CO and 937 μM O₂. Photodissociation of CO allows association of O₂ followed by a return to the CO form (ligand replacement reaction). Kinetics were measured at 25°C and recorded at various wavelengths and time scales as described (14, 37).

**CO kinetics.** To measure rates of CO association, laser-flash photolysis experiments were performed using the setup described above in O₂ kinetics. Samples of CO-ligated ferrous Mb were prepared in a sealed 4 × 10 mm quartz cuvette containing 1 ml of 100 mM potassium phosphate buffer, 1 mM EDTA, pH 7.0. The buffer was equilibrated with different CO concentrations (100–400 μM) obtained by mixing N₂ and CO gas using a High-Tech system (Brinkhorst, Venendael, The Netherlands). Protein was added to a final concentration of ∼5 μM. A 10-μl saturated solution of sodium dithionite was then added to the buffer to fully reduce the globin. The oxidation state, extent of CO binding, and heme concentrations were confirmed by UV-visible absorption spectra. Experiments were performed at 25°C.
Reactions of deoxy Mb1 and Mb2 with nitrite. The reactions of deoxy Mb1, rec-Mb1, and Mb2 (∼10 μM heme) with nitrite were investigated under pseudo-first-order conditions at varying sodium nitrite concentrations (0.1–0.83 mM) and in the presence of small amounts of sodium dithionite (36), as previously described (32). Before the addition of nitrite, the N2-equilibrated Mb sample was titrated anaerobically with a solution of dithionite (∼30 mM) until the absorbance spectrum indicated that the Mb was fully deoxy (<100 μM dithionite). Experiments were performed in 10 mM HEPES, 0.5 mM EDTA, pH 7.6, at 25°C. The reaction between carp deoxy Mb1 and nitrite (0.54 mM) was also investigated at pH values of 6.7–8.3. Nitrite solutions were freshly prepared every day, and the concentration was checked with the Griess assay (42).

Sulfhydryl reactivity. Reactive Cys (-SH/heme) of Mb1 and Mb2 was measured by reaction with 4,4′-dithiodipyridine (4-PDS), which is converted into 4-thiopyridone (4-Tp, ε = 19.8 M−1 cm−1 at 324 nm) upon reaction with free thiols (16). The molar ratio heme/4-PDS was 1:2 or 1:4. Other conditions were as previously described (21).

S-nitrosation and analysis of SNO samples. Carp Mb1 and Mb2 were S-nitrosated by reaction with S-nitroso Cys (Cys(–NO)) as described (21). To minimize heme oxidation in the reaction, the Mbs were equilibrated with pure CO. Cys-NO was added at a 10:1 Cys-NO/heme molar ratio, and the mixture was left to react for 5–10 min at room temperature and in darkness. Longer reaction times resulted in partial oxidation of the Mbs to the ferric (met) form. Excess Cys-NO was removed by desalting on a PD-10 column (GE Healthcare) equilibrated with 50 mM Tris, pH 8.3. All solutions were kept capped, on ice, and in darkness when not in use. The yield of SNO/heme was measured by the Saville assay (38), as described (21). O2 equilibria of Mb1-SNO and Mb2-SNO were obtained by measuring each saturation step in separate experiments, to minimize duration of light exposure of the samples and SNO decay, as described for trout Mb-SNO (21).

Peroxidase activity. H2O2 (∼20 μM) was added to oxy Mb1 and oxy Mb2 solutions (∼10 μM heme, 10 mM HEPEs, pH 7.5) at a 2:1 H2O2/heme molar ratio, and the decrease in absorbance at 416 nm was monitored using a HP 8543 UV-visible diode array spectrophotometer. To correct for autoxidation of the Mbs, absorbance of samples with water added instead of H2O2 was measured in parallel over time.

Statistics. Unpaired t-tests were performed to compare mean P50 and observed rates of Mb1 (native) and Mb2. Statistical significance was accepted at the 95% confidence interval (P < 0.05). Numerical values obtained in triplicates (n = 3) are given as means ± SD.

Molecular phylogeny. The duplicated carp Mbs were aligned with other teleost fish Mb protein sequences using CLUSTAL X as implemented in MEGA version 4.0 (44), and a maximum likelihood molecular phylogeny was constructed with PhyML 3.0 (18), using default settings. Branch support was estimated using bootstrap analysis with 1,000 pseudo replicates. Mb sequences of the following species were used [scientific names and National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) accession numbers in parentheses]: common carp Mb1 and Mb2 (Cyprinus carpio, P02204 and ABC69306), goldfish Mb1 and Mb2 (Carassius auratus, CA000405 and CA000406), zebrafish (Danio rerio, NP 001187526), milkfish (Chanos chanos, BAH22136), hilsa shad (Tenualosa ilisha, BAH90801), rainbow trout (Oncorhynchus mykiss, NP 001165333), Pacific mackerel (Scomber japonicus, Q9D9G9) and yellowfin tuna (Thunnus albacares, P02205). The last three species were used as outgroup to root the tree.

RESULTS

Expression and purification of recombinant carp Mb1 and Mb2 yielded two proteins with the expected molecular mass of ∼16–17 kDa as assessed by SDS-PAGE (Fig. 1, inset). Native carp Mb1 was successfully purified from contaminating hemo-
globin (Hb) by ammonium sulfate fractionation and FPLC gel filtration of heart homogenates (Fig. 1).

P50 values of native Mb1 purified from the carp heart and of rec-Mb1 were 1.0 ± 0.1 and 1.1 ± 0.1 Torr, respectively (25°C, pH 7.4), confirming identical properties of the two proteins. Mb2 had a significantly higher P50 of 1.7 ± 0.1 Torr (n = 3, P < 0.001) and therefore a lower O2 affinity compared with Mb1 under identical conditions (Fig. 2A). This difference in affinity was confirmed by O2 binding kinetics, showing that the ratio K (equilibrium dissociation constant) between koff and kon was 2.1 and 4.5 μM for Mb1 and Mb2, respectively (kinetic values are listed in Table 1), indicating the same approximately twofold increase in O2 affinity in Mb2 relatively to Mb1. Cooperativity values (n50) derived from the slopes of the Hill plots (Fig. 2B) were 1.0 ± 0.1, 1.0 ± 0.1, and 1.0 ± 0.01 for Mb1, rec-Mb1, and Mb2, respectively, consistent with the monomeric structure of both Mbs. Overall heats of oxygenation ΔH (kcal/mol) derived from the slopes of the van’t Hoff plots (Fig. 2C) for Mb1 and Mb2 were −15.7 ± 0.2 and −16.4 ± 0.1 kcal/mol, respectively, indicating similar temperature sensitivities. No significant effect on O2 affinity of pH was found for either Mb1 (pH 6.4: P50 = 1.2 ± 0.2 Torr; pH 8.3: P50 = 1.0 ± 0.1 Torr) or Mb2 (pH 8.3: P50 = 1.8 ± 0.1 Torr). O2 equilibria of Mb2 at pH 6.4 were not measurable because the absorbance signal was noisy and decreased over time, indicating that Mb2 was unstable at acidic pH. Similarly, addition of lactate had no apparent effect on the P50 of either Mb1 at pH 6.5 (10 mM lactate: P50 = 1.0 Torr; 100 mM lactate: P50 = 0.9 Torr) or Mb2 at pH 7.4 (10 mM lactate: P50 = 1.9 Torr; 100 mM lactate: P50 = 2.0 Torr).

The CO association rates of Mb1 and Mb2 were measured using laser flash-photolysis. CO binding to both Mbs showed biphasic kinetics that could be fitted by a double exponential function (not shown) yielding fast \([k_{on1,Mb1} = (4.4 ± 0.7) \times 10^6 \text{ M}^{-1} \text{s}^{-1}; k_{on1,Mb2} = (3.2 ± 0.2) \times 10^6 \text{ M}^{-1} \text{s}^{-1}]\) and slow \([k_{on2,Mb2} = (2.0 ± 0.4) \times 10^6 \text{ M}^{-1} \text{s}^{-1}; k_{on2,Mb1} = (0.9 ± 0.2) \times 10^6 \text{ M}^{-1} \text{s}^{-1}]\) bimolecular rate constants (Table 1). This kinetic heterogeneity suggests the existence of two different conformations at equilibrium of the recombinant proteins, possibly due to opposite orientations of the inserted heme. Mb2
has slightly higher association rate constants than Mb1, suggesting a greater accessibility of the heme pocket for CO. Along with these bimolecular rebinding rates, also a very fast, concentration independent rebinding phase was present ($k_{\text{gem,Mb2}} = 5.0 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$; $k_{\text{gem,Mb1}} = 1.1 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$). This phase is ascribed to the rebinding of photodissociated CO still within the protein matrix (geminate rebinding).

When nitrite was added to deoxy Mb1 or Mb2 under anaerobic conditions, the absorbance visible spectrum changed over time from that of deoxy Mb (Fe$^{2+}$) having a single peak at 555 nm, to that of NO-bound Mb (Fe$^{2+}$-NO) having peaks at 545 and 572 nm (data not shown). This follows the general scheme for nitrite reduction catalyzed by deoxy globins in the presence of dithionite (32), which reduces the met (Fe$^{3+}$) product to the deoxy (Fe$^{2+}$) form, which in turn binds the NO produced:

$$\text{Fe}^{2+} + \text{NO}^- + H^+ \rightarrow \text{Fe}^{3+} + \text{NO} + \text{OH}^- \quad (1)$$

$$\text{NO} + \text{Fe}^{2+} \rightarrow \text{Fe}^{2+/-\text{NO}} \quad (2)$$

Therefore, the Fe$^{2+}$-NO derivative is the only final heme product of the reaction. Observed rates ($k_{\text{obs}}$) were obtained by fitting absorbance traces at 555 nm to a single exponential function (Fig. 3A). Apparent second-order rate constants (from the plot of $k_{\text{obs}}$ as a function of nitrite concentration) were 5.3 ± 0.7 M$^{-1}$s$^{-1}$ and 1.8 ± 0.4 M$^{-1}$s$^{-1}$ for Mb1 and Mb2, respectively (Fig. 3B). Rec-Mb1 showed similar reaction rates to Mb1 (Fig. 3B). The dependency on pH of the reaction was investigated for Mb1. As the reaction stoichiometry requires one proton (Eq. 1), decreasing the pH by one unit would accelerate the reaction rate 10-fold. Accordingly, the slope of the fitted linear regression of log$k_{\text{obs}}$ vs. pH for Mb1 at 0.54 mM nitrite was −1.0 ($r^2 = 0.99$) (Fig. 3C).

Reaction with 4-PDS to measure reactive thiols showed that Mb1 and rec-Mb1 have $\sim$1.0 and Mb2 has $\sim$1.2–1.7 -SH/heme (data not shown), consistent with Mb1 having one and Mb2 two Cys residues in their amino acid sequences (13). S-nitrosation with CyS-NO yielded $\sim$0.4–0.6 and $\sim$0.3–0.4 SNO/heme for Mb1 and Mb2, respectively, suggesting that one Cys was partially S-nitrosated in both Mbs. After $\sim$4 h on ice, SNO/heme on Mb1 had declined to $\sim$55% of its original value, as measured by the Saville assay (not shown). The S-nitrosated Mbs showed almost identical O$_2$ affinity to the non-nitrosated Mbs (Fig. 4) with $P_{50} = 1.1 \pm 0.1$ Torr ($\Delta P_{50} = 1.2 \pm 0.2$) for Mb1-SNO and $P_{50} = 1.7 \pm 0.01$ Torr ($\Delta P_{50} = 1.0 \pm 0.02$) for Mb2-SNO at 25°C.

Because in vivo Mb will be in the oxy form after reoxygenation following a hypoxic period, we examined the reaction of oxy Mb1 and oxy Mb2 with a twofold molar excess of H$_2$O$_2$. The change in absorbance spectra over time indicated the two-electron oxidation of oxy Mb (Fe$^{2+}$-O$_2$) with a peak at 414 nm to ferryl Mb (Fe$^{4+}$-O) with a peak at 417 nm (Fig. 5A). Spectra showed isosbestic points at 527 and 585 nm, in agreement with previous results on the reaction of H$_2$O$_2$ with horse heart Mb (48). The ferryl form decayed spontaneously overnight to the ferric (Fe$^{3+}$) form (data not shown) (51). The spectral changes shown in Fig. 5A are consistent with the

Table 1. Equilibrium and kinetic values among myoglobins

<table>
<thead>
<tr>
<th>Species</th>
<th>$P_{50}$, Torr</th>
<th>$k_{\text{on,O}_2}$, $\mu$M$^{-1}$s$^{-1}$</th>
<th>$k_{\text{off,O}_2}$, s$^{-1}$</th>
<th>$K$, $\mu$M</th>
<th>$k_{\text{on,CO}}$, $\mu$M$^{-1}$s$^{-1}$</th>
</tr>
</thead>
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<tr>
<td>Common carp</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mb1 (SNO)</td>
<td>1.0 (1.1)</td>
<td>7.1</td>
<td>14.9</td>
<td>2.1</td>
<td>3.2</td>
</tr>
<tr>
<td>Mb2 (SNO)</td>
<td>1.7 (1.7)</td>
<td>16.6</td>
<td>75.2</td>
<td>4.5</td>
<td>4.4</td>
</tr>
<tr>
<td>Horse</td>
<td>0.8*</td>
<td>19*</td>
<td>17*–37*</td>
<td>1.9*</td>
<td>0.7*</td>
</tr>
<tr>
<td>Sperm whale</td>
<td>0.9*</td>
<td>19*</td>
<td>17*–10*</td>
<td>0.5*</td>
<td>0.7*</td>
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<tr>
<td>Yellowfin tuna</td>
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<td>nd</td>
<td>53*</td>
<td>1.5*</td>
<td>21</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>4.9</td>
<td>nd</td>
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Mb1 and Mb2 O$_2$ affinities ($P_{50}$), O$_2$ association ($k_{\text{on,O}_2}$), and dissociation ($k_{\text{off,O}_2}$) rate constants with the derived equilibrium constant ($K = k_{\text{off,O}_2}/k_{\text{on,O}_2}$), and CO association rate constant ($k_{\text{on,CO}}$) measured at 25°C compared with values for Mbs from other species. Values for Mb1-SNO and Mb2-SNO from common carp are indicated in parentheses. a, 22°C. b, 23°C. c, 20°C. nd, not determined.
reaction mechanism described for oxy-Mb involving prior O\textsubscript{2} dissociation and subsequent binding of H\textsubscript{2}O\textsubscript{2} to the ferrous heme as the rate limiting step (51):

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{4+} = \text{O} + \text{H}_2\text{O}
\]  

Absorbance traces at 416 nm were best fitted by a single exponential function (Fig. 5B), indicating that reactions were effectively first order in nature and that only one main reaction was present. The rates obtained from the fitted exponential showed that the reaction of H\textsubscript{2}O\textsubscript{2} with oxy Mb2, \(k_{\text{obs}} = (1.3 \pm 0.3) \times 10^{-3} \text{ s}^{-1}\), was significantly faster than that with oxy Mb1, \(k_{\text{obs}} = (0.7 \pm 0.1) \times 10^{-3} \text{ s}^{-1}\) (\(n = 3, P = 0.013\)) (Fig. 5B). Reaction kinetics with sub-stoichiometric amounts of H\textsubscript{2}O\textsubscript{2} were too slow to provide reliable rate values.

**DISCUSSION**

The discovery of a broad tissue expression of Mb1 and of a brain-specific Mb2 isoform in the hypoxia-tolerant common carp has suggested crucial cytoprotective functions of these proteins, especially of Mb2, linked to hypoxia adaptation and survival (13). Here we demonstrate that Mb1 and Mb2 have distinctive biochemical properties that suggest Mb2 may potentially reduce oxidative stress in the brain, particularly during reoxygenation after hypoxia.

Mb2 has a lower O\textsubscript{2} affinity than Mb1. We show here a significant difference in the O\textsubscript{2} affinities of the two carp Mb1-Mb2 having a lower affinity \(P_{50} = 1.7 \pm 0.1\text{ Torr}\) than Mb1 \(P_{50} = 1.0 \pm 0.1\text{ Torr}\) at 25°C. This difference in O\textsubscript{2} affinity is due to a fivefold higher O\textsubscript{2} dissociation rate \(k_{\text{off}}\) of Mb2 compared with Mb1, whereas the O\textsubscript{2} association rate \(k_{\text{on}}\) of Mb2 is only 2.3-fold higher than that of Mb1 (Table 1). The structural basis for the fast kinetics of Mb2 O\textsubscript{2} binding and dissociation (resembling that of yellowfin tuna Mb, Table 1) is currently under investigation. The faster O\textsubscript{2} kinetics of Mb2 also align with a faster CO binding kinetics than Mb1 (Table 1). The high \(P_{50}\) of Mb2 oxygenation argues against a major role for this protein in O\textsubscript{2} extraction from the blood Hb during hypoxia. In the hypoxic carp, the Hb O\textsubscript{2} affinity increases and may decrease to \(P_{50}\) values as low as 3.0 Torr due to a decreased concentration of erethrocytic GTP (46). Thus Mb2 would be able to extract less O\textsubscript{2} from the blood Hb during hypoxia than during normoxia. In contrast to Mb2, the \(P_{50}\) of carp Mb1 found here was similar to that typical of most Mbs isolated from heart and skeletal muscle of vertebrates, including fish (4, 29), and therefore compatible with facilitated diffusion and storage of O\textsubscript{2} under normoxia and hypoxia and not only in the heart, where Mb1 is present at high levels, but also in the liver and skeletal muscle, where Mb1 expression is upregulated during hypoxia (13). In general, the O\textsubscript{2} affinities of fish Mbs span a broader range than mammalian Mbs, with some fish species like trout \(P_{50} = 4.9\text{ Torr}\) and mackerel \(P_{50} = 2.0\text{ Torr}\) showing low-affinity Mbs. This probably reflects the greater variability of physiological conditions and requirement for oxygen in aquatic compared with terrestrial species.

We found no effect of varying pH or lactate concentration on the O\textsubscript{2} affinity of either Mb1 or Mb2, consistent with previous findings on fish Mbs (21, 29), but in contrast to one report of a lactate effect in horse and sperm whale Mb (15). Overall \(\Delta H\) values for carp Mb1 and Mb2 were \(-15.7 \pm 0.2\) kcal/mol and \(-16.4 \pm 0.1\) kcal/mol, respectively, indicating that the O\textsubscript{2} affinities of carp Mbs are less temperature-sensitive than in other fish Mbs, with \(\Delta H\) values between \(-18\) and \(-25\) kcal/mol (1, 29). A relatively low \(\Delta H\) would keep the O\textsubscript{2} affinity more constant in an environment where the temperature may vary substantially.

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**Fig. 3.** Kinetic traces and observed reaction rate versus nitrite concentration for the reaction of deoxy Mb1, Mb2, and rec-Mb1 with nitrite. A: representative absorbance traces at 555 nm for the reaction of ferrous Mb1 and Mb2 (\(-10\) \(\mu\text{M}\)) with 0.4 mM nitrite measured in 10 mM HEPES buffer, 0.5 mM EDTA, pH 7.6, 25°C, under anaerobic conditions. Fitting of data according to a monoexponential function (which gives the observed rate, \(k_{\text{obs}}, \text{s}^{-1}\)) is indicated. B: plot of \(k_{\text{obs}}\) for the individual reactions of ferrous Mb1, Mb2, and rec-Mb1 as a function of nitrite concentration. The second-order rate constant for the nitrite reductase activity is given by the slope of the linear regression. C: dependence on pH of the observed reaction rate of Mb1 with nitrite. All values are means ± SD.

**Fig. 4.** Representative O\textsubscript{2} equilibrium curves showing changes in the \(P_{50}\) of Mb1 and Mb2 when converted to Mb1-SNO (\(-0.42\) SNO/heme) and Mb2-SNO (\(-0.32\) SNO/heme), respectively (\(-100\) \(\mu\text{M}\) heme in 50 mM Tris, pH 8.3, 25°C).
**Mb2 is a faster peroxidase than Mb1.** Carp oxy Mb1 and Mb2 react with a twofold molar excess of H2O2 at rates (0.7 × 10⁻³ s⁻¹ and 1.3 × 10⁻³ s⁻¹, respectively) that are comparable to that of sperm whale oxy Mb measured under similar conditions (−2 × 10⁻³ s⁻¹; Ref. 51). That Mb2 reacts almost twice as rapidly with H2O2 compared with carp Mb1 indicates that it may have a special function in attenuating oxidative stress in the carp brain. With estimates of in vivo levels that may increase from 10⁻⁹ M under steady state to 10⁻⁷ M during ischemia and reperfusion (5, 23), H2O2 is the most abundant among ROS in tissues (20). The reaction of oxy Mb with H2O2 yields the two-electron oxidized ferryl Mb (47, 51), which is a potent oxidant and may have detrimental effects such as peroxidation of lipids (34). However, with Mb in excess of H2O2, as it would occur in vivo, ferryl Mb undergoes a synproportionation reaction with ferrous Mb yielding ferric Mb (48). In the cell, ferric Mb is reduced back to ferrous Mb by metmyoglobin reductase (19), thus making Mb available to undergo another redox cycle with H2O2 until all H2O2 is eliminated. In accordance with this chemistry, Flögel et al. (12) demonstrated that Mb effectively protects mice hearts from oxidative damage inflicted by added H2O2 and endogenous ROS generated during ischemia and reperfusion. As H2O2 reacts with unbound ferrous heme (51), the twofold faster reaction of Mb2 with H2O2 is in good agreement with the larger fraction of deoxy heme (1%) than in Mb1 (0.6%) in air, as derived from the O₂ equilibrium data. Thus the lower O₂ affinity of Mb2 relative to Mb1 provides a higher degree of deoxygenated heme and consequently a faster removal of excess H2O2. This could be particularly useful when high levels of H2O2 and other ROS are generated like, e.g., in the reoxygenation period after prolonged, severe hypoxia (2).

Intriguingly, neuroglobin (Ngb), an ancient globin expressed in the brain of vertebrates (3) and having neuroprotective functions (43), is also expressed in the carp brain (13). However, Ngb does not react with H2O2 (22), probably as a result of heme hexacoordination. Recent evidence indicates that the neuroprotective role of Ngb depends instead on its redox reactivity, and two models have been recently proposed (11, 25).

**Mb1 is a faster nitrite reductase than Mb2.** We found that the rate constant of the reaction with nitrite for carp deoxy Mb1 (5.3 ± 0.7 M⁻¹s⁻¹) corresponds well with that for horse deoxy Mb (6 M⁻¹s⁻¹) (24), for sperm whale deoxy Mb (5.6 M⁻¹s⁻¹) (45), and for rainbow trout deoxy Mb (5.5 M⁻¹s⁻¹) (32). Thus the rate of carp Mb1 is fully consistent with a physiological role of this Mb in NO generation from nitrite during hypoxia to protect tissues where Mb1 is expressed, such as heart and skeletal muscle, liver, and endothelial cells (6, 13) by inhibiting mitochondrial respiration. Perhaps important, fish appear to lack endothelial NO synthase (9) and may thus be more dependent than higher vertebrates on endothelial Mb1 to generate of NO from nitrite in their cardiovascular system. That deoxy Mb2 displays a significantly lower nitrite reductase activity (1.8 M⁻¹s⁻¹) than Mb1 (5.3 M⁻¹s⁻¹), argues against a major functional role for Mb2 in protecting the carp brain neurons from hypoxia through generation of NO.

**Effects of S-nitrosation.** Mb1 and Mb2 contain one and two reactive Cys, respectively, that are in part accessible to S-nitrosation by CysNO. Although the yield of S-nitrosation was similar to that of tuna Mb (39) and trout Mb (21), there was no effect of S-nitrosation on the O₂ affinity in either carp Mb1 or Mb2. This contrasts with trout Mb, where a similar extent of S-nitrosation was found to increase its O₂ affinity (21). Nevertheless, the Cys residues of both carp Mbs may still participate in cellular SNO homeostasis without oxygenation-linked allosteric control. Furthermore, the additional, externally positioned Cys residues of Mb2 (13) could also increase thiol-dependent redox buffer capacity during oxidative stress and improve cellular protection in those regions of the brain where Mb2 is expressed. Fish Mbs generally have a larger number of Cys residues than mammalian Mbs, which suggests that their importance in the regulation and maintenance of cellular redox state may exceed that in mammals (M. Berenbrink, unpublished observation).

**Evolution of Mb1 and Mb2.** Assessing the extent and direction of functional diversification of duplicated isoforms requires knowledge about the functional properties of the original, unduplicated protein as a starting point. It is thus of interest to establish when the duplicated Mbs first arose. Duplicated isoforms and widespread nonmuscle expression of Mb have also been found in goldfish, a close carp relative, whereas the genome of the more distantly related zebrafish (Danio rerio) reveals a single Mb gene (35) with broad tissue specificity, including the brain (6). This is in line with, but not proof of, an origin of the paralogous Mbs due to the postulated whole genome duplication event in a common ancestor of carp and goldfish (both tetraploid) after their divergence from the zebrafish lineage (diploid) (7). The present maximum likelihood molecular phylogeny clearly supports an origin of the duplicated Mb isoforms in a common ancestor of carp and goldfish after the divergence from the zebrafish lineage (Fig. 6A), although addressing the detailed mechanism of gene duplication awaits genomic carp sequences (10). Thus, using the P₅₀...
of recombinant zebrafish Mb (1.0 Torr, 25°C; 28) as indicative of the ancestral Mb function before duplication, suggests that reduced O₂ affinity of carp Mb2 is a newly evolved function of this brain-specific isoform that can be causally linked to its increased peroxidase activity (see above). The Mb1 and Mb2 paralogs of carp and goldfish show numerous, nonconservative, and often mutually exclusive, amino acid substitutions that are found in no other Mb (Fig. 6). This may indicate relaxing or changed selection pressures acting on different regions of the two paralogs after functional diversification and changes in tissue expression. While these structural changes are too numerous for unambiguous assignment to functional differences, we note a unique, nonconservative Lys to Gln substitution at position 82 (F3) in the heme linked F-helix of carp Mb2 that may be linked to the reduced O₂ affinity compared with carp Mb1 (Fig. 6B). Interestingly, the only other Mb not having Lys82 is goldfish Mb2 with His82 (Fig. 6B).

**Perspectives and Significance**

This study is the first to compare molecular functional characteristics of two Mb isoforms from the same organism and it has expanded the understanding of how Mb may evolve to generate novel isoforms with different tissue-specific functional roles. In the case of carp Mb2, the ability to rapidly scavenge H₂O₂ in vitro reveals a novel potential mechanism in the in vivo protection of the brain against oxidative stress that may add to the wide range of antioxidant defense strategies present in animals (31). In particular, polyploid species such as the carp appear as ideal model organisms to investigate the evolution of novel adaptive molecular mechanisms, as in the tolerance to oxygen deprivation. However, to distinguish between evolution of increased peroxidase activity in Mb2 after genome duplication requires data on this activity in zebrafish or other fish species with ancestrally nonduplicated Mbs. These aspects remain to be investigated.

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FUNCTION OF CARP MYOGLOBIN ISOFORMS 

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