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Oxygen affinity and amino acid sequence of myoglobins from endothermic and ectothermic fish

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Marcinek, David J., Joseph Bonaventura, Jonathan B. Wittenberg, and Barbara A. Block. Oxygen affinity and amino acid sequence of myoglobins from endothermic and ectothermic fish. *Am J Physiol Regulatory Integrative Comp Physiol* 280: R1123–R1133, 2001.—Myoglobin (Mb) buffers intracellular O_2 and facilitates diffusion of O_2 through the cell. These functions of Mb will be most effective when intracellular P_{O_2} is near the partial pressure of oxygen at which Mb is half saturated (P_{50}) of the molecule. We test the hypothesis that Mb oxygen affinity has evolved such that it is conserved when adjusted for body temperature among closely related animals. We measure oxygen P_{50}s tonometrically and oxygen dissociation rate constants with stopped flow and generate amino acid sequence from cDNA of Mbs from fish with different body temperatures. P_{50}s for the endothermic bluefin tuna, skipjack tuna, and blue marlin at 20°C were 0.62 ± 0.02, 0.59 ± 0.01, 0.58 ± 0.04 mmHg, respectively, and were significantly lower than those for ectothermic bonito (1.03 ± 0.07 mmHg) and mackerel (1.39 ± 0.03 mmHg). Because the oxygen affinity of Mb decreases with increasing temperature, the above differences in oxygen affinity between endothermic and ectothermic fish are reduced when adjusted for the in vivo muscle temperature of the animal. Oxygen dissociation rate constants at 20°C for the endothermic species ranged from 34.1 to 49.3 s^{-1}, whereas those for mackerel and bonito were 102 and 62 s^{-1}, respectively. Correlated with the low oxygen affinity and fast dissociation kinetics of mackerel Mb is a substitution of alanine for proline that would likely result in a more flexible mackerel protein.

kinetics; Scombroidei; oxygen binding; tunas

MYOGLOBIN (Mb) is a monomeric heme protein found in almost all cardiac and aerobic skeletal muscles of vertebrates (13). The oxygen affinity of Mb lies between those of hemoglobin and cytochrome oxidase and decreases with increasing temperature across all vertebrate taxa examined (1, 12). The primary roles of Mb are thought to be the facilitated diffusion of oxygen from capillary to mitochondria and oxygen storage, as in diving mammals (56, 57). Recent experiments in transgenic mice lacking Mb support the role of Mb in augmenting oxygen delivery to the mitochondria. Although Garry et al. (23) were unable to detect any effect of the knockout on whole animal aerobic performance, Gödecke et al. (27) demonstrated that several parameters of the oxygen delivery pathway are adjusted to compensate for the chronic loss of Mb.

Mb is one of most well-characterized proteins. Structurally, it is similar to the subunits of vertebrate hemoglobin and, like hemoglobin, can reversibly bind oxygen at the sixth coordination position of its heme iron. Mammalian Mb is composed of 153 amino acids and 8 α-helices labeled A through H (1). Teleost Mbs maintain the same characteristic globin-fold tertiary structure, but contain only 146 amino acids (54, 55) and lack the D-helix found in higher vertebrates (2). There have been many studies linking changes in Mb function to structural changes in the protein (17, 33, 39, 49). These studies have typically focused on the region of the protein surrounding the heme pocket, which is the site of oxygen binding. However, this region is highly conserved across all vertebrate taxa. Sequence comparison between functionally distinct proteins can help to identify residues, other than those comprising the heme pocket, involved in the subtle differences in proteins observed in nature.

Due to their diverse range of habitats, teleost fish provide an excellent system in which to study the natural variation of Mb function and to put that variation in the context of the physiological ecology of the animal. Nichols and Weber (38), working with scup, salmon, tuna, and mammalian Mbs, concluded that there was a correlation between oxygen affinity of Mb and body temperature. However, in their comparison of Mbs from several Antarctic fish species with those from yellowfin tuna, mackerel, and sperm whale, Cashon et al. (12) found no correlation between Mb oxygen affinity and body temperature. Differences in evolutionary history and physiological differences between species

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may explain the seemingly contradictory results from these two studies. This study minimizes differences attributable to phylogeny and differences in oxygen demand of muscle between species. With the exception of the blue marlin, which functions as the outgroup for the phylogenetic analysis, the fish used for this study are all members of the family Scombridae and share an active pelagic lifestyle (6) with high aerobic capacities in their slow-twitch axial muscle or heater organ (blue marlin) (15, 22, 35, 53).

Tunas are unique among teleosts in that they are capable of maintaining temperatures in their slow-twitch axial muscle significantly warmer than the surrounding water. Thermal excesses >20°C have been recorded for large Atlantic bluefin in waters as cold as 6°C (8, 10). Average body temperatures for Pacific bluefin tuna of 23–26°C in 15–18°C water have recently been reported (35). Tunas are able to maintain elevated core body temperatures due to the presence of counter-current heat exchangers that enable them to thermally isolate the core of their bodies and circumvent heat loss at the gills (10, 11). In billfish, endothermy is limited to their cranial cavity, where a region of the superior rectus extraocular muscle has evolved to act as a heater organ that warms the brain and eyes (4, 7). This tissue has lost its ability to contract but maintains a high aerobic capacity and Mb content (6). In contrast, the bonitos and mackerels are ectothermic fish that have little or no ability to maintain body temperatures significantly above that of the surrounding water.

We tested the hypothesis that the oxygen binding characteristics of scombroid Mbs will be correlated with body temperature. We determined oxygen binding affinities, ligand binding kinetics, and primary structures of Mbs from several species of endothermic and ectothermic fish of the suborder Scombroidei. Results indicated that, despite differences in physiological temperatures of 10°C or more between these fish, the oxygen affinities of Mbs from the endothermic fish, Pacific bluefin tuna (*Thunnus thynnus orientalis*), skipjack tuna (*Katsuwonus pelamis*), and blue marlin (*Makaira nigricans*), and the ectothermic, Eastern Pacific bonito (*Sarda chiliensis*) and Pacific mackerel (*Scomber japonicus*), are conserved when adjusted for the physiological temperatures of the animals. By comparing amino acid sequences from these proteins, we are able to highlight regions of the molecule that may be involved in functional differences between the proteins.

MATERIALS AND METHODS

Sample Collection

Mb was isolated from oxidative slow-twitch muscle from all fish examined. Bluefin tuna, skipjack tuna, and bonito were caught offshore of San Diego, CA, in 17–22°C water, and the mackerel were caught in Monterey Bay where the surface water temperature ranges from 11 to 17°C. Bluefin and skipjack tuna muscle samples were taken from wild-caught fish. Bonito and mackerel were maintained in tanks at 20°C at the Tuna Research and Conservation Center in Pacific Grove, CA, for several months before sampling. Blue marlin muscle was taken from a fish caught during the Big Rock Billfish Tournament off the coast of Morehead City, NC. All muscle samples were taken shortly after the fish were killed by pithing, frozen in liquid nitrogen, and stored at −80°C, with the exception of the blue marlin samples. Blue marlin muscle was sampled several hours after death and stored at −20°C until used. This resulted in greater proportion of metMb isolated from blue marlin than for the other species.

Protein Purification

Mbs were purified from all species following a protocol adapted from Wittenberg and Wittenberg (58). Oxidative muscle was thawed and homogenized in a Waring blender at low speed for 5–10 s in ice-cold buffer of 50 mM triethanolamine, 0.5 mM EDTA at pH 7.6 with 0.1 mM phenylmethylsulfonyl fluoride protease inhibitor. The homogenate was centrifuged at 10,000 *g* for 10 min, and ammonium sulfate was added to 65% saturation to the supernatant. After equilibration with gentle stirring for 20 min, the solution was centrifuged 10,000 *g* for 10 min. The supernatant was collected, and ammonium sulfate was added to 90% saturation. This solution was equilibrated as before and centrifuged at 30,000 *g* for 3 h. This entire procedure was carried out at 4°C, and pH was maintained between 8 and 8.5 with potassium hydroxide. The 90% saturation pellet was collected by carefully pouring off the supernatant, or, in some cases where the pellet floated on top of the solution, by carefully withdrawing the supernatant from below the precipitated Mb. The Mb paste was dialyzed overnight in a buffer of 50 mM potassium phosphate, 0.5 mM EDTA at pH 7.6. A 0.2-μm syringe filter was used to remove small particulates before separation over a Sephadex G-100 (Sigma) size exclusion column to remove residual hemoglobin and cytochrome *c*.

Ferric and ferrous Mbs from bluefin, skipjack, blue marlin, and bonito were further separated from each other over a CM-Sepharose Fast Flow (Sigma) ion exchange column in a gradient of monobasic potassium phosphate, pH 6.0, to dibasic potassium phosphate, pH 9. Ferric and ferrous Mb were collected and frozen at −80°C until used. This column did not separate mackerel Mb into oxy- and metMb fractions. Because of this, mackerel Mb was not purified beyond the size exclusion chromatography step (12). The presence and purity of Mb was followed throughout the purification procedure by SDS-PAGE and Western blotting with Sigma antibody to human Mb (M-7773) and by analysis of the absorption spectra from 400 to 650 nm.

Both ferric and ferrous Mbs were used in assays. Before use, ferric Mb was reduced with dithionite (42). The reduction was carried out by adding a few crystals of dithionite to 0.5 ml of concentrated metMb solution in phosphate buffer. The solution was mixed by inverting and then applied to a Sephadex G-25 column prepared in a 5-ml syringe barrel. The Mb solution was applied to the column within 60 s and centrifuged at high speed for 30 s. The oxyMb was collected in a 1.7-ml microfuge tube attached to the end of the syringe. This oxyMb solution was diluted in phosphate buffer for experiments. Control experiments demonstrated that reduction with dithionite did not affect the ligand binding behavior of Mbs.

Oxygen Binding Equilibria

Oxygen equilibrium curves were determined tonometrically as described in Antonini and Brunori (1). A volume of 2.5 ml of 60 μM Mb was used for each determination. Spectra

between 500 and 650 nm were recorded on a Hewlett Packard HP8453 UV-Vis Spectrophotometer after the addition of increasing amounts of room air with a gas-tight Hamilton syringe. An absorbance spectra between 500 and 650 nm was taken after each addition of air. The amplitudes of the first derivatives of the spectra were calculated at 540, 560, and 576 nm and were used to determine the percent oxygen saturation of the Mb solution. Tonometers were incubated in a temperature-controlled water bath at 20 and 25°C between injections of room air. Care was taken to minimize the time between the removal of the tonometer from the water bath and reading of the spectra (3–5 s). Oxygen equilibrium curves were determined in triplicate for each protein at 20 and 25°C. The partial pressure of oxygen at which Mb is half saturated (P_{50}) for mackerel Mb was calculated at 15°C using the Q_{10} between 20 and 25°C.

The effect of lactate on the oxygen affinity of scombroid Mbs was also assayed. A concentrated solution of L-lactic acid (Fisher) was added to Mb solutions to make 2.5 ml of 60 μM Mb plus 0, 5, 50, and 100 mM lactate. The pH of these solutions was adjusted to 6.8 with concentrated potassium hydroxide. Oxygen affinity was assayed as above at 20°C. The absorbance spectrum between 350 and 450 was also measured for each lactate concentration.

Oxygen Dissociation Rate Constants

Aerobic Mb solutions were diluted to ~5 μM with degassed buffer of 50 mM potassium phosphate, 0.5 mM EDTA at pH 7.6. This yielded fully oxygenated Mb in a low P_{O_2} buffer. A separate solution of 6 mg/ml dithionite in the same degassed buffer was used to trap oxygen on mixing. Oxygen dissociation rate constants were measured on a Gibson-Durrum stopped-flow spectrophotometer connected to a 486 Dell computer and analyzed with the OLIS Spectroscopy Operating System, version 14.09 software (On-Line Instrument Systems). Temperatures were measured in the chamber housing the drive syringes and mixing chamber of the stopped-flow apparatus and were maintained within ±0.5°C with a circulating water bath. The reaction was followed at 430 nm for 0.02–0.09 s. Curves were fit with nonlinear least-square approximations using a single exponential in all cases except for the oxygen dissociation rate constants for mackerel. These were clearly better approximated with a two-exponential model. Average rate constants represent three to seven determinations for each species at each temperature.

Cloning and Sequencing of Mb cDNA

Amplification of Mb cDNA. Total RNA was isolated according to TRI reagent protocol (Molecular Research Center) from slow-twitch muscle for all species. cDNA was produced with Boehringer Mannheim Superscript II reverse transcriptase from total RNA isolates. Partial-length Mb cDNA was amplified using degenerate primers made by combining information from published yellowfin tuna amino acid sequence (55) and a blue marlin Mb cDNA clone sequenced in our laboratory (J. Keen, unpublished data). A modified rapid amplification of cDNA ends protocol (30) was used to amplify sequence of the 5′-ends of the coding sequence and untranslated regions (UTR) of Mb cDNA from Atlantic bluefin and yellowfin tuna and the 3′-ends of the coding sequence and UTRs of Atlantic bluefin, yellowfin, frigate tuna, and mackerel. The template switching reverse transcriptase reaction and step-out PCR protocol from Matz et al. (36) were used to amplify the 5′-end of the coding sequence and UTR of mackerel Mb cDNA. Primers were then developed to amplify full-length cDNA fragments for cloning. Primers at the extreme 5′ (5′-AATCAGACGGGATATATTAC-3′)- and 3′ (5′-TTTTAAAGCAACAGAGAG-3′)-ends amplified full-length Mb cDNA from all scombroid species except mackerel and the entire coding region plus partial 3′- and 5′-untranslated regions from blue marlin. A different 5′-primer (5′-CAGAGATATCTCACTACTTTGC-3′) was paired with the 3′-primer above to amplify partial 5′-UTR, the entire coding region, and 3′-UTR of mackerel cDNA.

Cloning and sequencing. Full-length Mb cDNA PCR products were isolated on a 1% low melting point agarose gel and purified from the gel slice using Wizard PCR prep kit (Promega) and cloned into pGEM t-vector plasmids (Promega). The plasmids were then transformed into competent cells prepared by treating XL-Blue MRF′ *Escherichia coli* strain with 50 mM calcium chloride. Plasmid DNA was isolated using the Wizard Prep kit (Promega) for sequencing. Sequencing was performed on an Applied Biosystems, 373 automated sequencer. Full-length Mb cDNA clones were generated from multiple individuals for each species. Overlapping cDNA fragments generated from both ends of the clone were assembled to yield the complete sequence for the cDNA clones.

Sequence analysis. Mb amino acid sequence was translated from cDNA sequence using MacVector 4.1.1 and aligned with the ClustalW algorithm (52). Amino acid substitutions were mapped to the published crystal structure of yellowfin tuna Mb (2) and viewed using the RasMol freeware (46).

RESULTS

Oxygen Affinity

The oxygen binding curves determined at pH 7.6 and 20°C of Mbs isolated from the two ectothermic fish are right-shifted compared with the three endothermic fish, indicating lower oxygen affinity in Mbs from the ectothermic fish (Fig. 1*A*). Although the oxygen affinities of the three endothermic fish are very similar to one another, the oxygen affinity of bonito Mb is between those of endothermic fish and mackerel Mbs. The effect of temperature on oxygen affinity was also examined by estimating the enthalpy of oxygenation (ΔH) from measured oxygen affinities at 20 and 25°C using the integrated Van't Hoff equation (Table 1). Despite the differences in oxygen affinity at 20°C between the different Mbs, the temperature dependence of P_{50}s is similar for all species between 20 and 25°C (Fig. 1*B* and Table 1). Adjusting P_{50}s to the measured body temperatures of 25°C for the endothermic bluefin tuna, skipjack tuna, blue marlin (5, 10), and to the more ecologically relevant 20°C for bonito and 15°C for mackerel indicates that the oxygen affinities among all five species are similar at their physiological temperatures (Table 1).

In addition to the effect of temperature on oxygen affinity we also examined the effect of lactate on oxygen affinity for bluefin tuna Mb. We were unable to detect any effect of lactate concentrations of 0, 5, 50, and 100 mM in bluefin tuna Mb, which had oxygen P_{50}s of 0.69, 0.69, 0.66, and 0.64, respectively.

Phylogenetic Considerations

A 583-bp nucleotide sequence of cytochrome *b* (21) was used to construct a molecular phylogeny of the five

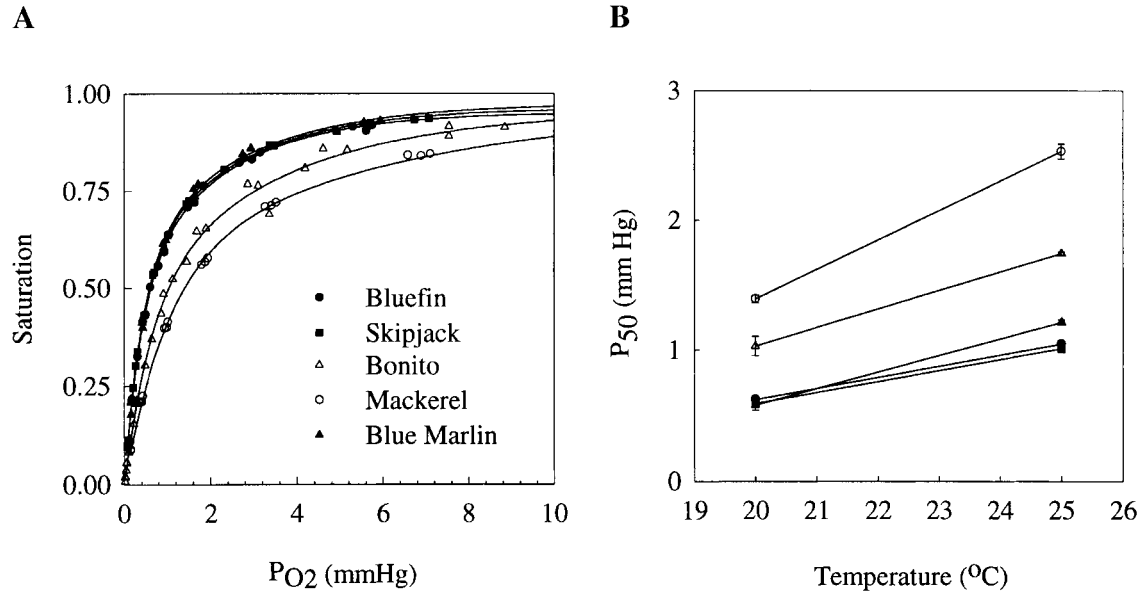

Fig. 1. Oxygen affinity for myoglobins from 5 species of scombroid fish. *A*: oxygen binding curves were determined tonometrically in triplicate at 20°C for each species. All data are shown in plots. Regressions were fit with a 4-parameter nonlinear regression model. *B*: oxygen P_{50}s calculated from oxygen binding curves at 20 and 25°C. Partial pressure of oxygen at which myoglobin (Mb) is half saturated (P_{50}s) were calculated from linear regressions of Hill plots of oxygen saturation data. Closed symbols represent endothermic fish. Open symbols represent ectothermic fish.

scombroid species included in this study (Fig. 2). Cytochrome *b* sequence was used, as opposed to Mb sequence, because it is independent of the character we are testing (i.e., Mb protein function). The maximum likelihood algorithm of PAUP* 4.0 (50) was used to determine the branching pattern. Values above the nodes indicate percent support for the given bifurcation out of 1,000 bootstrap replicates. Oxygen affinity at 20°C (P_{50}s given in the right column of Fig. 2) and the presence of endothermy (endothermic species in italics) are also indicated in Fig. 2. From this tree it is clear that high oxygen affinity is associated with the presence of endothermy in two distinct taxa, the blue marlin and the tunas, to the exclusion of ectothermic intermediate groups.

Table 1. *Oxygen affinity and enthalpy of oxygenation for myoglobins*

Species	Approximate T_b, °C	P_{50}, mmHg		ΔH, kcal/mol
		20°C	T_b	20–25°C
Bluefin tuna	25	0.62 ± 0.02	1.05 ± 0.02	−18.2
Skipjack tuna	25	0.59 ± 0.01	1.01 ± 0.03	−18.6
Blue marlin	25	0.58 ± 0.04	1.21 ± 0.02	−25.4
Bonito	20	1.03 ± 0.07*	1.03 ± 0.07	−18.1
Mackerel	15	1.39 ± 0.03*	0.76†	−20.7

Values are means ± SD. Partial pressure at which myoglobin is half saturated (P_{50}s) given in column 4 are at the physiological temperature (T_b) of each species. Enthalpy of oxygenation (ΔH) was calculated from the integrated Van't Hoff equation between 20 and 25°C. *Significant difference from endothermic fish ($P < 0.05$). †P_{50} for mackerel myoglobin at 15°C was calculated from the P_{50}s measured at 20 and 25°C for this species.

Kinetics

Oxygen dissociation rate constants (k_{off}) for mackerel Mb were significantly higher (Fig. 3*A*) and the oxygen affinity significantly lower than those from all other species at every temperature (Tukey-Kramer multiple comparison test from ANOVA $P < 0.05$). Results with bonito Mb were between those for mackerel and those for the endothermic species at each temperature (Fig. 3*A* and Table 2). This pattern is difficult to discern at lower temperatures, but becomes more apparent at the higher temperatures. Above 30°C it was difficult to

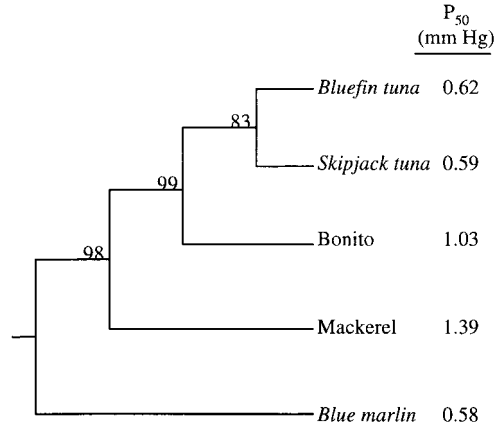

Fig. 2. Oxygen affinity mapped to molecular phylogeny determined with 583 nucleotides of cytochrome *b*. Sequences were aligned with ClustalW, and distances were calculated with maximum-likelihood algorithm using PAUP*. Numbers above branches represent bootstrap values with 1,000 replicates. Blue marlin was set as the outgroup. Endothermic species are in italics, and oxygen P_{50} at 20°C is shown for each species in the column to the *right*.

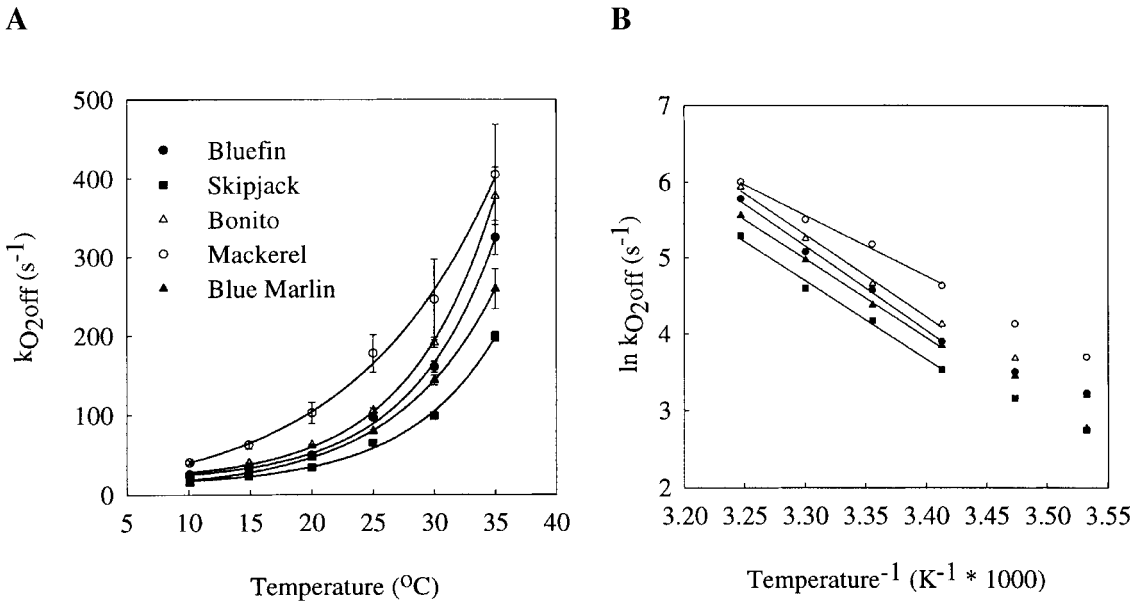

Fig. 3. Oxygen dissociation rate constants. *A*: dissociation rate constants plotted (kO_{2off}) vs. temperature in degrees Celsius fit with a 2-parameter exponential equation. Error bars are 1 SD. *B*: Arrhenius plots of data in *A*. The natural log of oxygen dissociation rate constants are plotted against the inverse of temperature multiplied by 1,000 in K. Linear regressions were fit to each species between 20 and 35°C.

consistently measure oxygen dissociation rate constants of mackerel Mb, as is evident by the large standard deviations of the points at 30 and 35°C for this protein. Second-order combination rate constants (k_{on}) were calculated from measured values of K_D and the dissociation rate constant at 20°C (Table 2). The combination rate constants were similar across all species.

The two-exponential analysis for the oxygen dissociation from mackerel Mb yielded one fast and one slow reaction that contributed equally to the total absorbance change of the reaction (data not shown). The faster rate corresponded to published values for another species of mackerel (12) and was similar to values for oxygen dissociation rate constants from the other scombroid species and was therefore taken to be the oxygen dissociation rate constant for Pacific mackerel in this study. The slower rate is likely due to the reduction of ferric Mb by dithionite.

Table 2. *Kinetics parameters of oxygen binding to myoglobins*

Species	K_D, μM 20°C	k_{off}, s^{-1} 20°C	k_{on}, μM/s 20°C	E_a, kcal/mol 20–35°C
Bluefin tuna	1.13 ± 0.04	49.3 ± 3.24	43.6	22.0
Skipjack tuna	1.08 ± 0.04	34.1 ± 0.35	31.7	20.4
Blue marlin	1.06 ± 0.07	46.6 ± 2.54	44.0	20.5
Bonito	1.88 ± 0.13	62.1 ± 1.46	33.1	20.0
Mackerel	2.53 ± 0.06	102 ± 13.5	40.5	15.9

K_D was calculated by multiplying the measured P_{50}s by the solubility of oxygen at 20°C (1.82 μM/mmHg). Association rate constants were calculated using the relationship $k_{on} = k_{off}/K_D$. Activation energies (E_a) for oxygen dissociation from myoglobin were calculated from the slopes of the Arrhenius plots in Figure 2*B*.

The Arrhenius plots of the oxygen dissociation data are nonlinear for the temperature range from 10 to 35°C for scombroid Mbs (Fig. 3*B*). To estimate the effect of temperature on the dissociation kinetics, linear regressions were fit for each species between 20 and 35°C. The activation energies (Table 2), which are directly proportional to the effect of temperature on a reaction, were calculated from each slope in Fig. 3*B* (48). Activation energies for these scombroid Mbs are similar with the exception of that for mackerel Mb.

Primary Structure

The amino acid sequences of scombroid myoglobins are presented with that of sperm whale myoglobin in Fig. 4. The numbering used is that for mammalian myoglobin to facilitate comparison with the literature. Similar to other teleost Mbs sequenced, scombroid Mbs contain 146 amino acids, with the exception of skipjack tuna, which have a deletion at position 52. Residues creating the hydrophobic lining of the heme pocket and those involved in stabilizing the heme (44) are highly conserved across all scombroids sequenced (Fig. 4). The three residues suggested by Giardina et al. (25) to be involved in binding lactate in sperm whale and horse Mbs, 47 (CD3), 64 (E7), and 67 (E10), are also conserved in all scombroid Mbs.

Despite the similarities between teleost Mbs, there are several amino acid differences among the scombroids that occur in otherwise conserved regions of the protein. In all teleosts sequenced to date, there is a proline at position 16 in the A-helix (44, 54, 55), which causes a kink in the helix (2). Skipjack tuna Mb is unique in having an alanine for proline replacement at position 16. In four of seven of the fish Mbs presented there is a cysteine residue at position 13 that presents

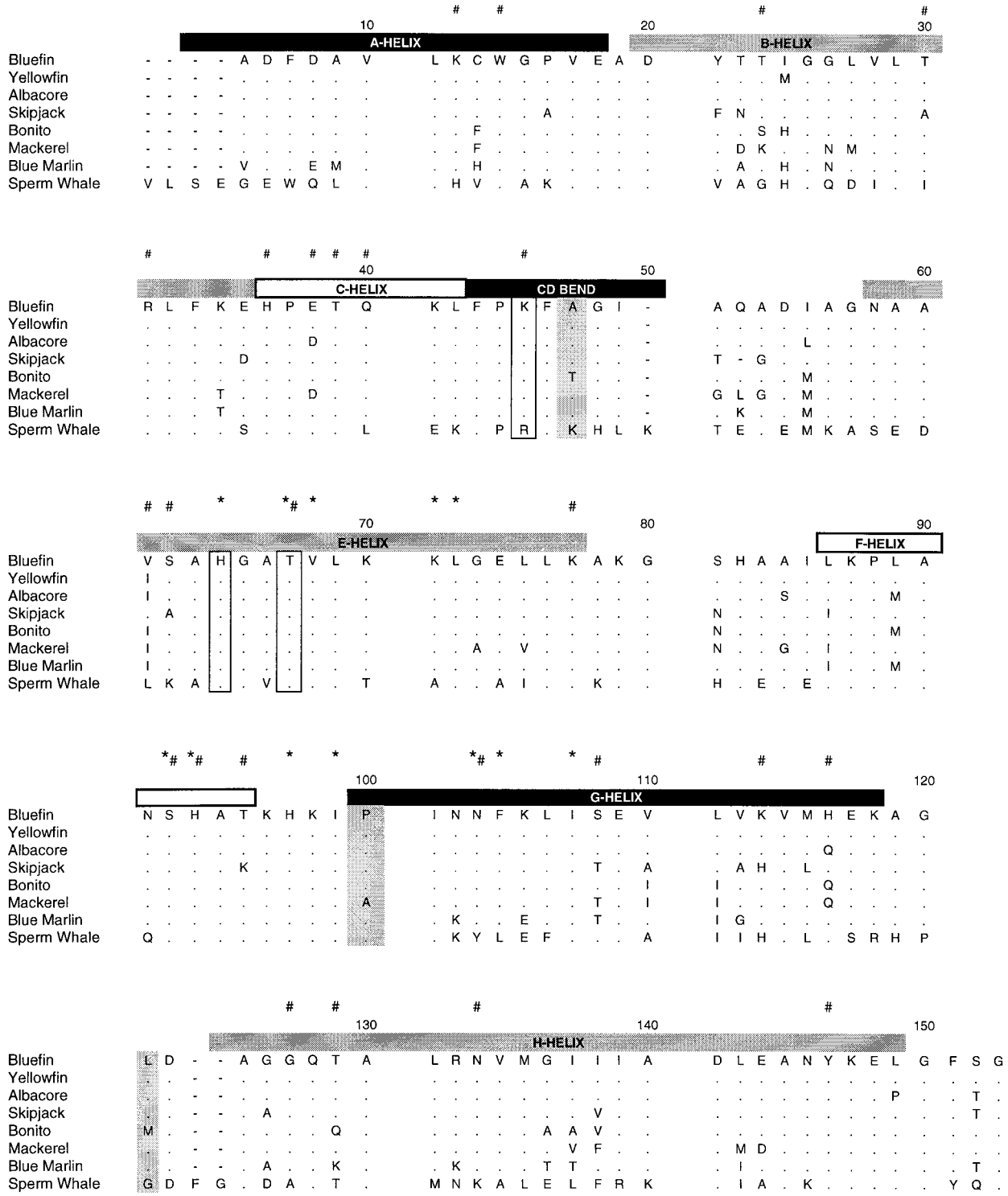

Fig. 4. Alignment of Mb amino acid sequences. The shaded bars above the sites indicate the residues making up the 7 α-helices and the bend between helices C and D (CD bend). Sperm whale Mb sequence from Ref. 18. Deletions are denoted by "–." Heme-contact residues and non-covalent intramolecular bonding sites in teleosts Mb are denoted by "*" and "#," respectively (2, 43). Sites in nonhelical regions where bonito or mackerel Mb differ from other teleosts are shaded. Suggested lactate binding residues are boxed.

a reactive sulfhydryl group on the exterior of the protein (35). A cysteine residue occupies position 13 in all other sequenced teleost myoglobins, including several Antarctic icefish and carp. In bonito, mackerel, and blue marlin Mbs, position 13 contains a phenylalanine, phenylalanine, and histidine, respectively.

The two Mbs that possess low oxygen affinity, bonito and mackerel, have a few differences in relatively con-

served regions of the molecule. In bonito Mb, alanine is replaced by threonine and leucine by methionine at positions 47 (CD5) and 121, respectively. In mackerel Mb, position 73 and 75 in the E-helix show two amino acid replacements, glycine to alanine and leucine to valine, respectively. There is also a unique substitution of proline to alanine at the NH_2-terminal end of the G-helix. This exchange of alanine for proline is also observed in carp Mb. The only site where both bonito and mackerel Mbs are different from teleost Mbs with high oxygen affinity (notothenioids, tunas, and marlin) is position 23. In all other scombroids there is a threonine at this site, whereas bonito and mackerel contain a serine and lysine, respectively. This site is involved in intramolecular hydrogen bonding in yellowfin tuna Mb (2). Both serine and lysine are capable of hydrogen bonding.

DISCUSSION

We have demonstrated that Mb oxygen affinity is correlated with in vivo aerobic muscle temperatures for the endothermic and ectothermic fish in this study. All five species are active pelagic predators representing a range of body temperatures and thermal strategies. The oxygen affinities of Mbs at 20°C from all three endothermic fish were similar and significantly higher than for the two ectothermic species. The measured Mb P_{50}s from tuna and marlin are close to published values for yellowfin tuna (12, 38), although they are lower than those reported for Atlantic bluefin tuna (P_{50} = 0.9 mmHg at 20°C) (45). The Pacific mackerel used in this study, *Scomber japonicus*, has a higher oxygen affinity than the Atlantic mackerel species used by Cashon et al. (12), *Scomber scombrus* (P_{50} = 3.7 at 24°C), which inhabits colder waters than the Pacific mackerel (14). Although it is possible that there were some changes in Mb function in vivo due to captivity in the mackerel and bonito, these are unlikely to have affected the oxygen binding characteristics of the proteins used in this study. Because purified proteins were used in our assays, the only route for acclimation that would affect our results would be covalent posttranslational modification. Although we did not explore this possibility, there is no evidence for posttranslational modification of any Mb.

Reasonable estimates of body temperature are between 23 and 30°C for the tunas and marlin (5, 10, 35), 17 and 22°C for bonito, and 10 and 17°C for mackerels. When measured at any given temperature, the P_{50}s for the endothermic and ectothermic fish differ significantly from one another. However, when oxygen P_{50} values are adjusted to physiologically relevant temperatures, the differences between these species are reduced or disappear (Table 1). When data presented in Table 1 are combined with the phylogenetic tree presented in Fig. 2, it becomes clear that high oxygen affinity is associated with the occurrence of endothermy in two distinct taxa, the blue marlins and the tunas, to the exclusion of the ectothermic groups.

A relatively conserved oxygen affinity of Mb from closely related fish may be linked to the proposed role of Mb in facilitating diffusion of oxygen from capillary to mitochondria. A necessary condition for the facilitated diffusion of oxygen by Mb is that Mb must be partially desaturated somewhere in the myocyte (59). In vivo experiments clearly demonstrate that during exercise, Mb is partially desaturated (volume average) in working mammalian muscle (37, 41). For Mb to be partially desaturated, the partial pressure of oxygen must be only a few torr. At these low oxygen partial pressures, small changes in oxygen affinity of the Mb can have large effects on the fractional saturation. A difference in P_{50} of only 0.5–1 Torr could mean the difference between 50% saturation and nearly complete desaturation or fully saturated Mb in muscle working near their maximum sustained rate of oxygen consumption.

Previous studies have resulted in conflicting conclusions about the correlation between body temperature and Mb oxygen affinity in teleosts. In Mbs from salmon, sculpin, and yellowfin tuna, oxygen affinity was observed to correlate with body temperature (38). However, in a comparison between Antarctic teleosts and yellowfin tuna, there was no correlation between these two variables (12). The species included in the previous studies are phylogenetically disparate species that fill different ecological niches. The large phylogenetic distances and metabolic differences between these groups add confounding factors to this comparison. In this study, differences between the species other than body temperature have been minimized by selecting closely related fish with similar active pelagic lifestyles. At physiological temperature, maximal tissue level aerobic demand is similar for bluefin, skipjack, bonito, and mackerel as indicated by maximal citrate synthase activities of the slow-twitch axial muscle (15, 22, 35). For the blue marlin, the maximal citrate synthase activities in the heater organ are higher than those from the slow-twitch axial muscle of the scombrid species (53).

Evolutionary Implications

A correlation between two characters could be explained by either a functional relationship between the characters or by the evolutionary relationships between the organisms studied (29). For Mb oxygen affinity and muscle temperature, this second possibility can be addressed by mapping both endothermic ability and Mb oxygen affinity onto a phylogenetic tree produced using an independent character, in this case cytochrome *b* nucleotide sequence (Fig. 2). Previous work has shown that endothermy has evolved independently in the billfish and tuna lineages (6). High oxygen affinity maps to the two independent endothermic branches, but the ectothermic taxa, which are more closely related to the tunas than are the billfish, possess Mbs with low oxygen affinity (Fig. 2). Therefore, the correlation between oxygen affinity and body temperature in the scombroid fish is not due to the phylogenetic relationship of the species. To further investigate the role of temperature in the evolution of Mbs, it

will be useful to examine the relationship between body temperature and oxygen affinity of Mbs in other groups that show similar variation in thermoregulatory ability, such as the lamnid sharks (9).

Mb in the hearts of tunas and billfish most likely functions at or near ambient temperature, whereas conditions in the aerobic axial and ocular muscles, respectively, can be 10–20°C higher. This temperature heterogeneity in tuna and billfish will present conflicting selective pressures on the Mbs of these fish. To investigate the possibility of multiple Mb isoforms that are differentially expressed in different tissues, we sequenced Mb cDNA from slow-twitch axial muscle and heart from Atlantic bluefin tuna, two highly aerobic tissues that are likely to see the greatest difference in temperature within an individual. These transcripts were identical to one another (data not shown), indicating that there is only one Mb isoform in bluefin tuna.

Because there is no evidence of multiple Mb isoforms, at least in bluefin tuna, it appears that Mbs in these species will be subject to conflicting selection pressures in regard to temperature. It is reasonable to assume that Mb will play the largest role in the tissue in which it is expressed in the highest concentrations. It follows from this that the function of Mb in these same tissues will have the greatest impact on the performance of the animal. We therefore suggest that the conditions in the tissues that have the highest Mb concentrations, which are the tissues with elevated temperatures in both tunas and blue marlin (3, 16), will be the site of the greatest pressures driving the evolution of Mbs.

Oxygen Binding Kinetics

Oxygen affinity (P_{50}), expressed in terms of oxygen partial pressure (Table 1), may also be expressed in molar terms (K_D) (Table 2) as the concentration of free, dissolved oxygen required to give half saturation at the temperature of measurement. K_D in turn, is given by the ratio of the oxygen dissociation and combination rate constants, $K_D = k_{off}/k_{on}$. Accordingly, the oxygen combination rate constants, which were not measured, may be approximated from the measured values of K_D and k_{off}. We have noted that the Mb oxygen affinity at a fixed temperature (i.e., 20°C) is high for endothermic species and low for ectothermic species (Fig. 2) and that the different oxygen affinities of the diverse species examined become more similar when adjusted to the probable temperatures of the working muscles. Our results suggest that the oxygen equilibrium constant (K_D^{-1}) is more strongly conserved than the two kinetic constants. Put differently, the oxygen equilibrium constant is the parameter that is responsive to selection in Mbs in these species.

Due to the nonlinearity of the Arrhenius plots for O_2 dissociation, activation energies were only calculated from 20 to 35°C. These estimates indicate that the activation energies of scombroid Mbs, with the exception of mackerel Mb, are in the same range as those of mammalian Mbs (1). Nonlinear Arrhenius plots have been observed for oxygen dissociation rate constants of Mbs from several Antarctic teleosts, yellowfin tuna, and Atlantic mackerel over the range of 0–20°C (12). The nonlinear Arrhenius plots could indicate multiple reactions in the system with different temperature dependencies or an effect of temperature on the conformation of the protein (31). Nonlinear Arrhenius plots of ligand dissociation kinetics have been reported for other fish Mbs (12). The dissociation of oxygen from Mb is a multistep pathway involving *1*) the separation of the heme and ligand, *2*) the movement of the ligand in the distal pocket, and *3*) the escape of the ligand from the distal pocket to the solvent (26). A difference in the activation energy of any one or multiple steps in this pathway could explain a curvilinear Arrhenius plot.

Primary Structure

Despite the conservation of oxygen affinity between bluefin, skipjack, and blue marlin, there are several features of the amino acid sequence of skipjack tuna Mb that are unique among teleosts. One is the deletion at position 52 in the unstructured region of the teleost molecule that forms the D-helix in mammalian Mbs. Whereas skipjack tuna is the only species in which this site is deleted, it shows a considerable variation in other teleosts, containing charged, polar, and nonpolar residues. Another unique feature in skipjack tuna Mb that is likely to have structural consequences is the proline to alanine substitution at position 16. In yellowfin tuna, a proline in this position causes a kink in the A-helix (2). This proline is present in all other teleosts except carp (44). In carp, position 16 is occupied by a glycine residue, which can also have helix destabilizing properties (47). Despite these differences, skipjack tuna Mb has almost identical oxygen affinity and similar kinetics to bluefin and blue marlin Mbs.

Correlation of Amino Acid Sequence with Oxygen Affinity

It is impossible to unambiguously link variation in amino acid sequence between Mbs in this study to differences in oxygen affinity. However, there are amino acid differences in bonito and mackerel Mbs that occur in regions of the molecule that are highly conserved in other scombroids. Two such differences occur in regions of the molecule between helices. These are the replacement of the nonpolar leucine with a polar methionine residue at position 121 in bonito and the replacement of a proline with an alanine at position 100 in mackerel. The proline to alanine difference in mackerel is particularly interesting in that it lies at the NH_2-terminal end of the G-helix next to a heme-contact residue. The only known vertebrate Mb, other than mackerel and carp, that is variant at this site is Mb of the possum, which has a serine for proline replacement (43). Proline adds rigidity to the peptide backbone by preventing rotation around its α-carbon (47). Replacement of proline by alanine provides a

greater degree of freedom in the α-carbon-nitrogen bond and could therefore allow greater movement of the G-helix. This difference is interesting in light of the low oxygen affinity and fast oxygen dissociation kinetics of mackerel Mb compared with that of other teleosts and mammals (12). Unfortunately, there is no information on either the oxygen affinity or kinetics of carp Mb.

The regions between the helices may act as hinges between the helices and affect the conformational flexibility of proteins (24). These regions have been proposed to be important sites for the modulation of conformational flexibility (20, 24). One barrier to oxygen dissociation from Mb is the escape of oxygen from the distal pocket. Amino acid substitutions in the hinge regions may contribute to increased flexibility of Mbs from bonito and mackerel that would make the conformational changes in the protein contributing to oxygen escape more likely and could result in an increase in the dissociation rate constant. Such an increase in the oxygen dissociation rate constant is particularly evident in mackerel Mb. Another effect of increased conformational flexibility is the increase in the entropy of the protein because it is free to assume a greater number of conformational states (19). Greater entropy in the ligand-free protein would likely result in an increase in the entropy change (ΔS), i.e., a larger negative ΔS, on the formation of the ligand-protein complex (19). Given an equal change in enthalpy (ΔH) of ligand binding, a larger negative ΔS will result in a lower negative free energy (ΔG) of ligand binding according to $\Delta G = \Delta H - T\Delta S$, resulting in lower oxygen affinity. Therefore, the proline to alanine substitution in mackerel Mb may be consistent with the lower oxygen affinity observed for this protein from both a kinetic and thermodynamic perspective.

Non-Heme Ligand Binding Sites

Analysis of the primary structure of scombroid Mbs also raises several interesting questions about non-heme ligand binding sites. Giardina et al. (25) demonstrated an allosteric modification of the oxygen affinity of sperm whale and horse Mbs by lactate. They proposed a lactate binding site at E7-CD3-E10. These residues also make up part of the pathway for entry of oxygen into the distal pocket of Mb. If this is the site for lactate binding to Mb, the conservation of these residues from mammalian to teleost Mbs would suggest that lactate would also be an allosteric modifier of teleost Mbs. Neither the spectral shifts in the Soret peak nor differences in oxygen binding affinity associated with lactate binding in mammalian Mbs were observed in bluefin tuna Mb. A recent report also failed to show allosteric modification of Emperor penguin Mb, despite the conservation of the suggested anion binding residues (51). It appears that if lactate is indeed an allosteric modifier of sperm whale and horse Mbs, conservation at the residues E7-CD3-E10 is not sufficient to produce the effect.

Functional Consequences of Cys(9)

Another possible site of non-heme ligand interaction in teleost myoglobins is the cysteine at position 9. Cysteine residues contribute a reactive sulfhydryl group to proteins. Most fish and reptile Mbs possess at least one cysteine residue, whereas no birds and only a few mammals contain cysteine in their Mbs. Among the teleosts, species as diverse as the Antarctic notothenioids, carp, and tunas have a conserved cysteine at position 13 in the A-helix (Fig. 4), whereas the non-tuna scombroids, bonito, mackerel, and blue marlin, lack this cysteine. The sulfhydryl group at position 13 is exposed to the exterior of the molecule and is therefore able to react with factors in the cytoplasm (35). One main function of cysteine residues in proteins is the formation of disulfide bonds, which covalently anchor the tertiary structure of proteins or join multiple polypeptides to form functional multimeric proteins. Because only one cysteine is present in the Mb molecule, the only avenue for formation of disulfide bonds is dimerization. Elution of all scombroid Mbs from a size exclusion column indicates that these Mbs exist as monomeric proteins despite the presence of a reactive sulfhydryl group (data not shown).

The reactivity of the sulfhydryl group also gives cysteine the propensity to react with nitric oxide (and other strong oxidizers). It is interesting to note that the three species lacking the cysteine at position 13 also contain more methionine residues than any other myoglobin, with the exception of Emperor penguin Mb (51). Methionine, like cysteine, is able to react with strong oxidizers and may protect the protein from oxidative damage (34). Free cysteine is known to react with nitric oxide in cells to form the nitric oxide donor nitrosocysteine. The exposure of the sulfhydryl of Cys(9) to the exterior surface of teleost Mbs may allow this protein to be S-nitrosylated at this site, thereby buffering the pathogenic effects of nitric oxide and preserving its bioactivity (28) and modulating its multiple effects on muscle contraction and metabolism (32, 40). However, the absence of Cys(9) in the more primitive scombrids is difficult to reconcile with this hypothesis.

Perspectives

This study provides evidence for adaptive variation in the O_2 affinity and temperature dependence of Mbs resulting in the conservation of O_2 affinity in vivo in teleosts with different body temperatures. Conservation of Mb P_{50} near the P_{O_2} in a heavily working muscle fiber is critical to modulation of intracellular P_{O_2} gradients and maintenance of Mb in a partially saturated state. Partially saturated Mb provides a pathway for the facilitated diffusion of O_2 through the muscle fiber. Buffering of intracellular P_{O_2} gradients and the facilitated diffusion of Mb are most significant at high O_2 demand when intracellular P_{O_2} is low. Hence, small changes in the O_2 affinity of Mb in vivo would lead to large differences in Mb oxygen saturation and efficacy of O_2 buffering and transport, an important adaptive

mechanism among species having different muscle temperatures. Amino acid sequence analysis of these Mbs suggests that amino acid substitutions in regions of the molecule outside of the heme pocket can have a large effect on the function of the protein. This supports the concept that point mutations and small changes at critical sites in an enzyme or protein can have a major effect on the functional properties of that protein. In this case, such small changes provide simple energetic and evolutionary mechanisms for maintaining functional flexibility of oxygen homeostasis in scombrid fish muscles.

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mRNA sequences for the myoglobin proteins presented here have been submitted to the National Center for Biotechnology and Information GenBank database. The accession numbers are AF291831–AF291838 for Atlantic bluefin tuna, albacore tuna, blue marlin, eastern Pacific bonito, Pacific mackerel, northern Pacific bluefin tuna, skipjack tuna, and yellowfin tuna, respectively.

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