JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization of Bullet Tuna Myoglobin with Reference to the Thermostability−Structure Relationship

NOBUHIKO UEKI,[†,§] CHAU-JEN CHOW,[‡] AND YOSHIHIRO OCHIAI*,[†]

Laboratory of Aquatic Molecular Biology and Biotechnology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo, Tokyo 113-8657, Japan, and Department of Seafood Science, National Kaohsiung Marine University, Nam-Tzu District, Kaohsiung, Taiwan, ROC

Myoglobin (Mb) was isolated from bullet tuna (*Auxis rochei*) skeletal muscle and characterized from the viewpoint of the thermostability−structure relationship. Differential scanning calorimetry (DSC) measurement showed that the thermostability of bullet tuna Mb was the lowest among all the scombridae fish Mbs so far examined. The highest value (72.8 °C) of melting temperature (T_m) was obtained at pH 6.52. α-Helical content at 10 °C was 34.5%, clearly lower than that of horse Mb (55.3%). The amino acid sequence was then deduced by cloning cDNA which encodes bullet tuna Mb. Bullet tuna Mb consisted of 147 amino acids, and the sequence identity was very close to that of skipjack (*Katsuwonus pelamis*) Mb (91.8%). A few amino acid substitutions, which could be involved in stability difference of Mb, were recognized. By mass spectrometry of lysyl endoproteinase digest of Mb, the N-terminus was found to be acetylated like that of other fish Mbs.

KEYWORDS: Myoglobin; thermostability; bullet tuna; cDNA cloning; amino acid sequence; CD; DSC

INTRODUCTION

Myoglobin (Mb) is a relatively compact, globular protein whose backbone structure consists of eight α-helical segments designated A through H, as revealed for the first time by Kendrew et al. on sperm whale Mb (*1*). The heme is contained in a hydrophobic 'heme pocket' and binds directly to the imidazole group of proximal histidine and to that of distal histidine through an oxygen coordinate binding (*2*). As far as fish Mb is concerned, a crystal structure of yellowfin tuna (*Thunnus albacares*) Mb has been solved (*3*). Its tertiary structure is similar to that of sperm whale Mb (*4*), though the tuna Mb lacks segment D. The N-terminal residue is valine or glycine in mammalian Mbs (*5*−*7*) but is varied in fish Mbs (*8*−*10*). The N-terminus is acetylated in bluefin tuna (*Thunnus thynnus*), skipjack (*Katsuwonus pelamis*) (*11*), and yellowfin tuna Mbs (*12*).

The color changes of Mb depend on the degree of oxidation of heme iron, namely on the composition of deoxyMb, oxyMb, and metMb, showing different visible absorption spectra. Accumulation of metMb derived from oxyMb, by oxidation of heme iron from a ferric to a ferrous form, results in deterioration of the meat quality, because the brownish color of metMb is enhanced. Previous studies showed that metMb is more susceptible to denaturation compared with the other forms, and progression of color darkening of tuna meat through freezing and thawing is faster than in unfrozen meat (*13*, *14*).

Fish Mbs are quite unstable than the counterparts of higher vertebrates and thus autoxidize and aggregate even under mild conditions (*15*, *16*). Chow (*17*) investigated the effects of pH on the denaturation profiles in concentrated guanidine hydrochloride and autoxidation rate for scombridae fish Mbs and found that lower stability was closely related to higher autoxidation rate. Other studies also showed that differences in stability exist among those Mbs as observed with thermal denaturation profile and autoxidation rate (*18*−*21*). Because scombridae fish have a high amount of Mb (*22*−*24*), the color change of Mb would directly affect their commercial values. Thus, detailed investigation for stability of Mb is essential for the quality control of these fish species.

Bullet tuna (*Auxis rochei*) is a scombridae pelagic species and is distributed along coastal waters nearly worldwide. In Japan, the annual catch is around 20000∼30000 tons, and this fish is important for a material of fish stick, which gives a peculiar dark-colored thick soup. Bullet tuna Mb may be related to coloration of this soup, because the ratio of dark muscle is comparatively high in this fish. Moreover, this species has recently been recognized as a good material for fish sauce, which is also characterized by a high 'a' value (redness), a nice flavor, and a lower level of salinity compared with those made from the other fish species (*25*). Despite such characteristics, bullet tuna still remains a kind of underutilized species and has a potential to be eaten raw, frozen stored, or processed for human consumption. One of the key factors for effective utilization is the stability of Mb as described above. Under these backgrounds,

* To whom correspondence should be addressed. Tel: +81-3-5841-7521. Fax: +81-3-5841-8166. E-mail: aochiai@mail.ecc.u-tokyo.ac.jp.
† The University of Tokyo.
‡ National Kaohsiung Marine University.
§ Present address: Laboratory of Peptide Biosignal Engineering, Mitsubishi Kagaku Institute of Life Sciences, Machida, Tokyo 194-8511, Japan.

10.1021/jf050261y CCC: $30.25
Published on Web 05/19/2005

Table 1. Sequence of Primers Used for cDNA Cloning

primer	sequence
MF1	5′-TGAAGTG(C/T)TGGGGTCCAGT-3′
MR1	5′-GAT(A/G)(C/G)CCATCACGTTCCTCA-3′
Ma5R1	5′-CGAGTCCAGCCTTCTCTTG-3′
Ma5R2	5′-GCAGCTCTCCAAGTTTCTTC-3′
Ma5R3	5′-GAACAGCTTCTGGGTATCAG-3′
Ma3R1	5′-CTGAAGGCCAAAGGCAATC-3′
Ma3R2	5′-GCACAAGATTCCCATTAATAAC-3′

we thought it is necessary to investigate the characteristics of bullet tuna Mb, of which no information is available at present.

In this study, to determine the properties of bullet tuna Mb, attempts were made to isolate this hemeprotein and characterize it from the viewpoint of thermal stability as measured by circular dichroism (CD) spectrometry and differential scanning calorimetry (DSC), with special reference to pH dependency and compared with those of several scombridae fish Mbs reported to date. Then, by cloning of cDNA-encoding Mb, the amino acid sequence was deduced and compared with those of other fish Mbs to explore the amino acid substitution(s) which could cause stability differences. Posttranslational modification of the N-terminus, which would also affect the stability, was also analyzed by MALDI-TOF mass spectrometry of the proteolytic fragments.

MATERIALS AND METHODS

Materials. Fresh specimen of bullet tuna was kindly provided from Dr. Seiji Akimoto (Kanagawa Prefectural Fisheries Research Institute, Japan). The specimens were stored at −80 °C until used for the experiments. Horse Mb as a control was purchased from Sigma Chemical (St Louis, MO). The subsequent procedures were performed at 0−4 °C unless otherwise stated.

Purification of Mb. Mb was purified essentially as reported previously (*26*). Briefly, the dark (slow skeletal) muscle was excised and extracted with 2 volumes of distilled water. After centrifugation, the filtrate was subjected to ammonium sulfate fractionation in the range of 50−80% saturation. The precipitate was dissolved in a small amount of water and dialyzed overnight against 50 mM Tris-phosphate (pH 8.0). The dialyzate was applied to gel filtration using a Sephadex G-100 column (26 mm × 100 cm; Amersham Biosciences Corp., Piscataway, NJ), equilibrated with 50 mM Tris-HCl (pH 8.0), at a flow rate of 60 mL/h. Elution patterns were monitored by the absorbance at 280 and 540 nm. The purity of Mb was checked by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE, 17.5% gel).

Differential Scanning Calorimetry (DSC). DSC was performed with a MicroCal VP-DSC differential scanning microcalorimeter (MicroCal Incorp., Northampton, MA) as reported previously (*26*). Protein concentration was adjusted to 0.49−0.69 mg/mL in 50 mM Na-phosphate (pH 7.0). DSC scans were carried out in triplicate at a rate of 60 °C/h in a temperature range of 10−90 °C. DSC data were analyzed using a software package (Origin) developed by MicroCal Inc. After minimization by appropriate computer programs, the values for the thermal transition, including the midpoint of melting temperature (T_m) and the molar excess heat capacity (ΔC_p), were obtained. For the pH dependency experiments, Mb was dialyzed against 50 mM Na-phosphate of various pH values (final pH 5.01−7.97), respectively.

Circular Dichroism (CD). CD measurement was carried out with a Jasco J-700W spectropolarimeter using a 0.2 mm water-jacketed cylindrical cell in a temperature range of 10−85 °C with the increment of 5 °C (10−60 °C) or 2.5 °C (60−85 °C). Wavelength for measurement was in the range of 240−195 nm. Determination of α-helical content was performed according to Yang et al. (*27*) with 200 μg/mL of Mb in 10 mM Na-phosphate (pH 7.0) containing 0.15 M KCl. The measurements were performed in triplicate.

Isolation of RNA and Synthesis of First Strand cDNA. Total RNA was extracted from fast skeletal muscle of bullet tuna using ISOGEN (Nippon Gene, Tokyo, Japan), and mRNA containing poly(A) tail was purified using Oligotex-dT30 Super (Takara, Otsu, Japan) according to the manufacture's protocol. First strand cDNA was synthesized using an aliquot of 5 μg of the total RNA. The *Not*I oligo dT primer (5′-AACTGGAAGAATTCGCGGCCGCAGGAA $(T)^{18}$-3′) was used to initiate first strand cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen Corp., Carlsbad, CA) in 50 mM Tris-HCl (pH 8.3), containing 75 mM KCl, 3 mM $MgCl_2$, 500 μM dNTP, and 10 mM dithiothreitol. The reaction was carried out at 42 °C for 1 h and was stopped by heat inactivation at 70 °C for 15 min.

cDNA Cloning of Bullet Tuna Mb. cDNA cloning was carried out as reported previously (*26*). Nucleotide sequences of primers used for polymerase chain reaction (PCR) are shown in **Table 1**. The locations and combinations of primers are indicated in **Figure 1**. Primers MF1 and MR1 were designed with reference to the highly conserved regions of the nucleotide sequences of cDNAs encoding Mb (corresponding to Ala9-Pro13 and Arg126-Ile131) from bluefin tuna, albacore, yellowfin tuna (*28*), and Nototheniidae fish (*Champsocephalus gunnari* and *Cryodraco antarcticus*) (GenBank accession numbers AF291831, AF291832, AF291838, U71054, and U71056, respectively). After PCR products were subcloned into the pGEM-T Easy vector (Promega, Madison, WI), nucleotides were sequenced by an ABI PRISM 3100 DNA sequencer (Applied Biosystems, Foster City, CA) using BigDye Terminator Cycle Sequencing Kit Version 3 (Applied Biosystems).

Primers Ma5R1, Ma5R2, Ma5R3, Ma3R1, and Ma3R2 for rapid amplification of cDNA ends (RACE) were designed based on the internal sequence of bullet tuna Mb cDNA obtained (**Figure 1**). In 3′ RACE, PCR was performed with the first strand cDNA as a template with primers Ma3R1 and Ma3R2 and with an abridged universal amplification primer (AUAP; 5′-GGCCACGCGTCGACTAGTAC-3′). PCR was carried out at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. The final extension step was performed at 72 °C for 5 min. 5′ RACE was performed using 5′ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen Corp.). First strand cDNA was synthesized from the total RNA with primer Ma5R1. Subsequently, PCR was carried out with primers Ma5R2 and Ma5R3 at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The final extension step was performed at 72 °C for 5 min.

Subcloning and nucleotide sequencing of both strands were performed according to the above methods. On the basis of the obtained nucleotide sequences of bullet tuna Mb cDNA, the amino acid sequence was deduced. Phylogenetic tree was drawn based on the nucleotide sequences of Mb cDNAs of bullet tuna and other species (*6*, *26*, *28*−*32*) by the neighbor-joining method using the PHYLIP program.

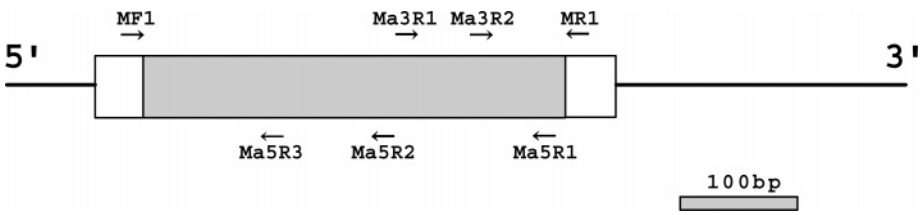

Figure 1. Schematic diagram of cDNA encoding bullet tuna myoglobin. The boxed area stands for a coding region. The lines indicate the noncoding regions, and the meshed area indicates the internal nucleotide sequence preliminarily determined using the primers MF1 and MR1 in the present study. The arrows indicate the positions of primers used. Refer to **Table 1** for the nucleotide sequences of primers.

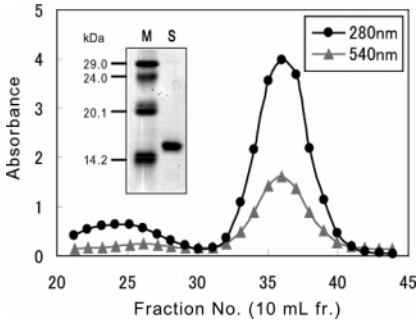

Figure 2. Gel filtration of bullet tuna myoglobin using a Sephadex G-100 column (26 mm × 100 cm) equilibrated with 50 mM Tris-HCl (pH 8.0). The inset shows SDS–PAGE pattern of purified myoglobin. M, mol. wt. markers (SDS-7); S, purified bullet tuna myoglobin. Five micrograms of purified myoglobin was applied. 17.5% gel.

Alignment of deduced amino acid sequences was performed using the Clustal W program.

N-Terminal Analysis. After heme was removed from Mb according to Takeda et al. (*33*), the molecular mass of apomyoglobin was measured by MALDI-TOF mass spectrometry using Voyager-DE STR (Applied Biosystems) with sinapinic acid as a matrix. Purified bullet tuna Mb was digested with 1/200 (w/w) endoproteinase Lys-C (Wako, Osaka, Japan) in 50 mM Tris-HCl (pH 8.0) for 1 h at 20 °C and desalted using an ODS column ($ZipTip_{18}$; Millipore, Billerica, MA), and subsequently MALDI-TOF mass spectrometry analysis was performed using α-cyano-4-hydroxycinnamic acid (Sigma) as a matrix. The peptide fragment of *m*/*z* 921.4, which was supposed to be subjected to acetylation of N-terminus, was isolated by HPLC using ODS-120A column (2.0 mm × 15 cm; TOSOH, Corp., Tokyo, Japan). Elution was carried out with a linear gradient of acetnitrile from 5% to 80% in the presence of 0.05% trifluoroacetic acid. The purified peptide was analyzed by post-source decay (PSD) mode of Voyager-DE STR using 2,5-dihydroxybenzoic acid (Wako) as a matrix. Theoretical values of product ions were calculated by MS-digest program of ProteinProspector on UCSF Mass Spectrometry Facility based on the deduced amino acid sequence of bullet tuna Mb.

Other Analytical Methods. Protein concentration was determined by BCA Protein Assay Kit (Pierce, Rockford, IL) using horse Mb as a standard, according to the manufacturer's protocol.

SDS−PAGE was performed by the method of Laemmli (*34*) using 17.5% gel. The standard molecular weight marker kit was purchased from Sigma (SDS-7).

RESULTS

Isolation of Myoglobin. As shown in **Figure 2**, Mb was purified from the dark muscle of bullet tuna. Fractions No. 35-37 were pooled and used in the following experiments as purified Mb (inset of **Figure 2**).

DSC Measurement. Investigation of pH dependency of the thermostability revealed that T_m was the highest (72.8 °C) at pH 6.52 and the lowest (64.9 °C) at pH 5.01 (**Figure 3**A). In the pH range from 7.03 to 7.97, a slight decline of T_m value was recognized (71.7 to 69.4 °C). In contrast, in the pH range lower than 5.74, T_m values largely decreased (71.9 to 64.9 °C) (**Figure 3B**).

The thermostability at pH 7.0 was compared with those of other scombridae fish species Mbs (T_m values of skipjack, bigeye tuna, bluefin tuna, and yellowfin tuna were 79.9, 75.7, 78.6, and 78.2 °C, respectively) by DSC measurement in 10 mM Na-phosphate (pH 7.0) containing 0.15 M KCl (*26*), taking horse heart Mb as a control. The value of bullet tuna Mb was 75.0 °C.

CD Measurement. Ellipticity values at 222 nm ($[\theta]_{222}$) increased as temperature was raised (**Figure 4**). The value at

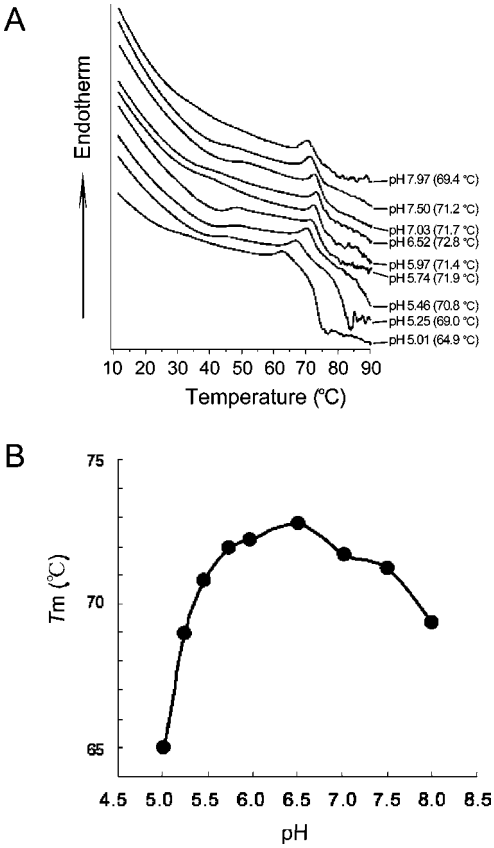

Figure 3. Effect of pH on DSC patterns (**A**) and the T_m values (**B**) of bullet tuna myoglobin. The actual pH values of sample solutions are indicated on the right in A, and T_m values are indicated in the parentheses. DSC was performed in 50 mM Na-phosphate (pH 5.01–7.97). Heating rate was 60 °C/h in the temperature range of 10–90 °C. Protein concentration was 0.49–0.69 mg/mL.

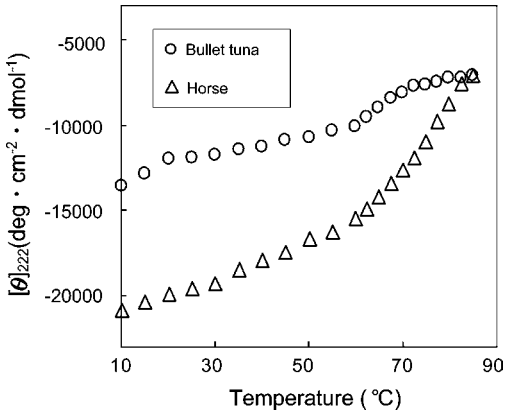

Figure 4. Effects of temperature on the mean residue ellipticity at 222 nm ($[\theta]_{222}$) of bullet tuna myoglobin compared with that of horse myoglobin as a control. CD spectrometry was performed in 10 mM Na-phosphate (pH 7.0) containing 0.15 M KCl in the temperature range of 10–85 °C with the increment of 5 °C (10–60 °C) or 2.5 °C (60–85 °C). Protein concentration was adjusted to 200 μg/mL.

10 °C was −13 800 for bullet tuna Mb, much higher than that of horse Mb (−22 100). Clear increment in mean residue ellipticity was recognized in the temperature range of 60−72.5 °C compared with those in the temperature range of 20−55 °C.

cDNA Cloning of Bullet Tuna Mb. An open reading frame of bullet tuna Mb cDNA contained 444 nucleotides, encoding 147 amino acids (**Figure 5**). The coding region was preceded by 5′ noncoding region of 75 base pair (bp) and was followed

```
AAAGAAGAATTTTGCTGTAATCAGACAGGATATATTACTACATTGCAGATCTAGATTTCTCCATCTCCTCAAATC   75

ATGGCTGACTTTGACGCAGTTCTGAAGTGTTGGGGCCCAGTGGAGGCGGACTTCAACACCGTTGGAGGCATGGTT  150
 M  A  D  F  D  A  V  L  K  C  W  G  P  V  E  A  D  F  N  T  V  G  G  M  V    25

CTGGCCCGTTTATTCAAAGACCACCCTGATACCCAGAAGCTGTTCCCCAAATTCGCTGGCATTGCCGCAGGTGAC  225
 L  A  R  L  F  K  D  H  P  D  T  Q  K  L  F  P  K  F  A  G  I  A  A  G  D    50

CTTGCCGGTAACGCGGCTGTTGCTGCTCACGGTGGCACTGTGCTGAAGAAACTTGGAGAGCTGCTGAAGGCCAAA  300
 L  A  G  N  A  A  V  A  A  H  G  G  T  V  L  K  K  L  G  E  L  L  K  A  K    75

GGCAATCACGCTGCCATCATAAAACCACTGGCAAACAGCCATGCCACTAAGCACAAGATTCCCATTAATAACTTC  375
 G  N  H  A  A  A  I  I  K  P  L  A  N  S  H  A  T  K  H  K  I  P  I  N  N  F   100

AAGCTGATCACTGAGGCCCTTGTGCATGTCATGCAAGAGAAGGCTGGACTCGACGCTGCTGGGCAGACAGCCCTG  450
 K  L  I  T  E  A  L  V  H  V  M  Q  E  K  A  G  L  D  A  A  G  Q  T  A  L   125

AGGAACGTGATGGGTATCGTCATCGCTGACCTTGAGGCCAACTACAAAGAGCTGGGCTTCACTGGCTGAGGTCAC  525
 R  N  V  M  G  I  V  I  A  D  L  E  A  N  Y  K  E  L  G  F  T  G  *          147

ATATGTCATGCCACTGTGTCGGACAGCAGACAGGAACATTTTCCACCAGCAAGTGCACATTTTAAAAGTTAGTTT  600
GCATAGGGTTTTTTTCTGTTATTGTGTCCACTCCTGTAACTGCTCAGTAATAAACATATTTCATATTTCTTGGCT  675
TGGCTAACAGGATGTAAACATCACCATATTGTACCCTGTTGTCACTTATTACCAATAAAACTCTCTGTTGCTTTA  750
AAAAAAAAAAAAAAAA                                                              766
```

Figure 5. Nucleotide and deduced amino acid sequences of bullet tuna myoglobin cDNA. The initiation codon is in bold-faced letters, and the termination codon is indicated by an asterisk. Amino acid numbers are indicated by bold-faced and italicized ones on the right. DDBJ/EMBL/GenBank accession no. for this sequence is AB154423.

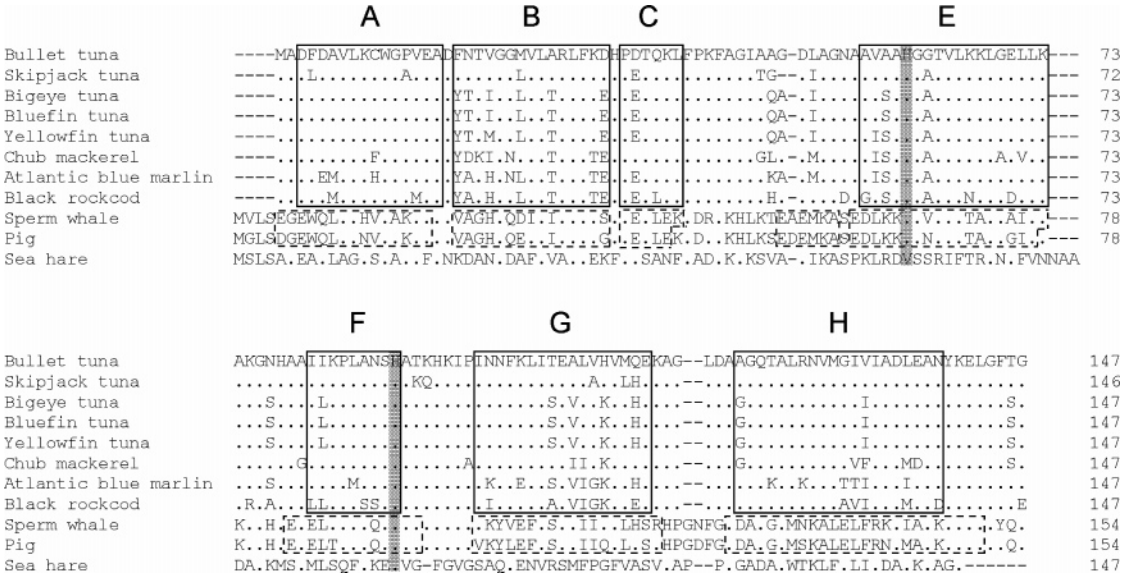

Figure 6. Alignment of amino acid sequence of bullet tuna myoglobin with those of other scombridae fish species. Amino acid residues identical to those of bullet tuna myoglobin are indicated with dots, and the gaps are indicated by dashed lines. Boxes contain the α-helical segments A, B, C, E, F, G, and H. Segment D is missing in fish myoglobins. Heme-binding histidine residues are shaded. For pig and whale myoglobins, the α-helical segments A through H are contained in dashed boxes. Accession numbers for the cited sequences are as follows: skipjack, AF291837; bigeye tuna, AB104433; bluefin tuna, AF291831; yellowfin tuna, AF291838; chub mackerel, AF291835; Atlantic blue marlin, AF291833; black rockcod, U71058; pig, M14433; sperm whale, J03566; sea hare, AB003277.

by 3′ noncoding region of 247 bp. DDBJ/EMBL/GenBank accession no. for this sequence is AB154423.

Homology of amino acid sequence between bullet tuna and skipjack Mbs showed the highest level (amino acid sequence identity was 91.8%) among those of Mbs so far reported (**Figure 6**, **Table 2**). The primary structure of bullet tuna Mb was found to be highly conserved, when compared with those of albacore, bigeye tuna, bluefin tuna, and yellowfin tuna (the sequence identities being 86.4, 85.7, 85.7, and 85.0%, respectively). However, chub mackerel *Scomber japonicus* and Atlantic blue marlin *Makaira nigricans* Mbs showed less homology, with the amino acid sequence identities being 81.0 and 76.9%, respectively, though several conservative regions, for instance, Thr36-Ile46 and Ala86-Ile95, were recognized. On the other hand, amino acid sequence identities of bullet tuna Mb with those of nototheniidae fish (sea raven *Hemitripterus americanus*, humped rockcod *Gobionotothen gibberifrons*, and black rockcod *Notothenia coriiceps*) were at relatively high levels (74.8−76.1%), but those with mammals (human *Homo sapiens*, pig *Sus scrofa*, horse *Equus caballus*, sperm whale *Physeter macrocephalus*, and Norway rat *Rattus norvegicus*) and a mollusk (sea hare *Aplysia juliana*) were low (41.5−44.9% and 16.6%, respectively).

When the amino acid sequence of bullet tuna Mb was compared in detail with those of other scombridae fish, a characteristic substitution of Ala62Gly (Ala62→Gly62) was disclosed. In addition, bullet tuna and skipjack Mbs shared the following substitutions from other scombridae fish Mbs:

Table 2. Identities of Amino Acid Sequence of Bullet Tuna Myoglobin with Counterparts of Other Species

species	identity (%)
skipjack	91.8
albacore	86.4
bigeye tuna	85.7
bluefin tuna	85.7
yellowfin tuna	85.0
Pacific bonito	81.0
chub mackerel	81.0
Atlantic blue marlin	76.9
sea raven	76.1
humped rockcod	74.8
black rockcod	74.8
human	44.9
pig	44.2
horse	42.9
sperm whale	41.5
Norway rat	41.5
sea hare	16.6

[a] Accession numbers of cited sequences are a follows: albacore, AF291832; Pacific bonito, AF291834; sea raven, AY029587; humped rockcod, U71057; black rockcod, U71058; human NM_005368; horse, P01288; Norway rat, AF197916; sea hare, AB003277. The numbers the other species are described in the legend of **Figure 6**.

Tyr18Phe, Thr27Ala, Glu32Asp, Ser58Ala, and Lys109His. Similarly, bullet tuna and chub mackerel Mbs had substitutions in common such as Glu35Asp and His112Gln. On the other hand, distal and proximal histidine residues that bind heme were conserved throughout all the species Mbs as in **Figure 6**.

In the phylogenetic tree based on nucleotide sequence, bullet tuna Mb was included in the scombridae fish group and located in the vicinity of skipjack Mb (**Figure 7**).

N-Terminal Analysis. Isoelectric point calculated by using the deduced amino acid sequence was 9.61. Molecular mass was 15497 as calculated from deduced amino acid composition, and 15407 as determined by mass spectrometry. In mass spectra of the lysyl endoproteinase digest, the peak of *m*/*z* 1010.2 corresponding to the fragment of MADFDAVLK located at the N-terminus, was not detected, but the peak of *m*/*z* 921.4 corresponding to the N-terminal acetylated fragment of ADFDAVLK (its theoretical value is *m*/*z* 921.0) was recognized instead (data not shown). **Figure 8** shows the mass spectra of product ions produced by the precursor ion of *m*/*z* 921.4. The actual values of product ions almost corresponded to the theoretical values (**Table 3**).

DISCUSSION

The T_m value of bullet tuna was the highest at around pH 6.5 (72.8 °C), and in the alkaline pH range, a slight decline of T_m values was observed. In the acidic pH range, especially lower than 5.74, T_m values decreased dramatically. This pH nearly matches the point of protonation of imidazole groups, the p*K*a of which is 6.0. Such instability may be partly involved in acceleration of oxidation at low pH and coincided with the results of the pH-dependent oxidation of bovine serum albumin (*35*). The above result was also in good agreement with the pH-dependent stability of tuna Mb through freezing and thawing as measured by autoxidation rate (*36*).

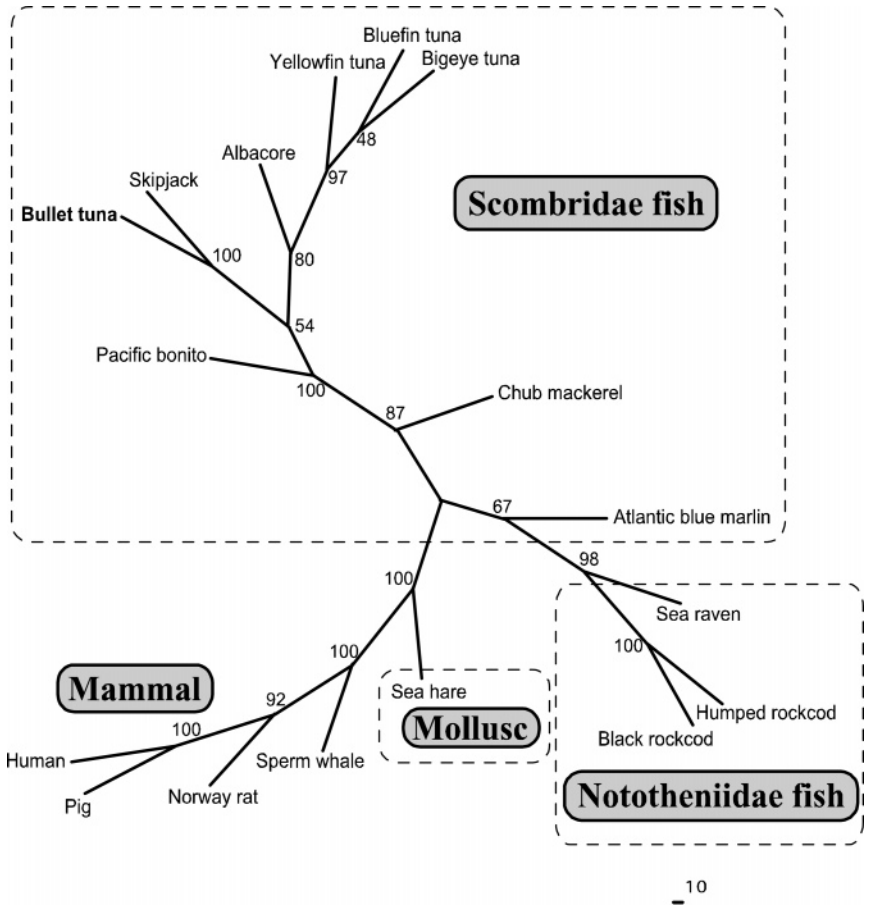

Figure 7. Phylogenetic tree based on the nucleotide sequences of myoglobin cDNAs by the neighbor-joining method. Numbers on the branches are the bootstrap confidence levels calculated from 1000 replicate analyses. Refer to the legend of **Figure 6** and the footnote of **Table 2** for the accession numbers of cited sequences. The scale is shown in percent accepted mutations.

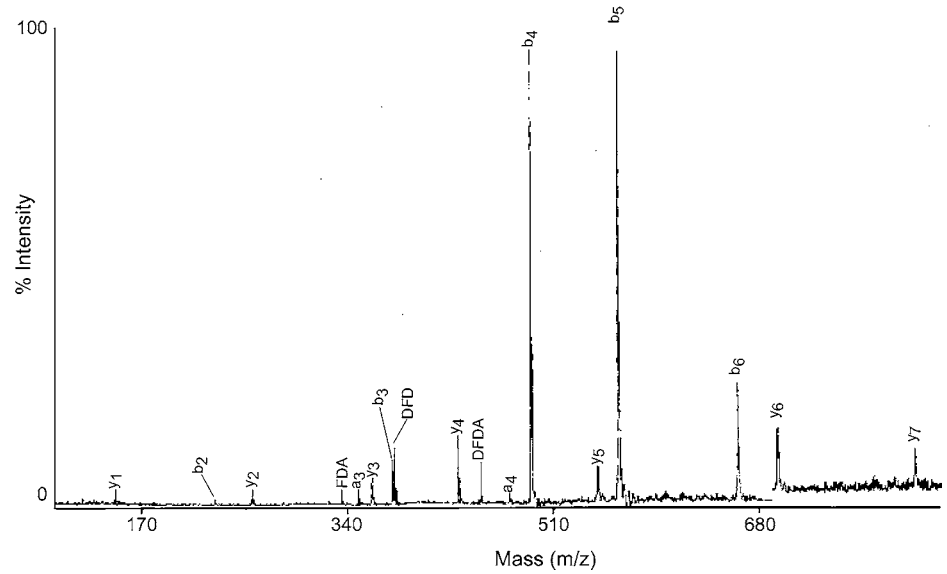

Figure 8. MS-MS spectra of precursor ion 921.4 (*m*/*z*) by PSD analysis. Assigned product ions are indicated on the top of the peaks.

Table 3. Actual Values of MS-MS Spectra from Precursor Ion (*m*/*z* 921.4) and Theoretical Values of Product Ions Assuming N-Terminal Acetylation of Peptide ADFDAVLK

ion species	actual value (*m*/*z*)	theoretical value (*m*/*z*)
a_2	ND[a]	201.1
a_3	348.6	348.2
a_4	463.8	463.2
a_5	ND	534.2
a_6	ND	633.3
a_7	ND	746.4
b_2	229.1	229.1
b_3	376.9	376.2
b_4	491.5	491.2
b_5	563.0	562.2
b_6	661.9	661.3
b_7	ND	774.4
y_1	147.2	147.1
y_2	260.0	260.2
y_3	359.7	359.3
y_4	431.0	430.3
y_5	545.3	545.3
y_6	692.6	692.4
y_7	807.5	807.4

[a] ND; not detected.

The T_m value of bullet tuna Mb (75.0 °C) was lower than that of horse Mb (84.2 °C) and the lowest among those of all the scombridae fish Mbs examined (75.7−79.9 °C). These results suggested that thermostability of bullet tuna Mb is also lower than that of the mammalian counterpart. DSC was performed two times sequentially for each sample, but in the second scan, no endothermic peak could be observed (data not shown).

As a result of cDNA cloning of bullet tuna Mb, its primary structure was found to resemble those of other scombridae fish Mbs. Phylogenetic analysis based on cDNA nucleotide sequences revealed that bullet tuna Mb belongs to the cluster of scombridae fish Mbs, especially close to skipjack Mb. This result was consistent with the highest conservation level of primary structure with skipjack Mb (91.8%) among all the species so far examined (**Table 2**). Sequence identity with other scombridae species Mbs was 76.9−86.4%. In contrast, the sequence identities were quite low with mammalian and molluscan Mbs. However, both the distal and proximal histidine residues were conserved among teleosts and mammals. Incidentally, distal histidine residues are known to be replaced by glutamine or valine in shark *Galeus nipponensis* (*37*) and some aplysiidae (*Dolabella auricularia*, *Aplysia kurodai*, *A. juliana*, and *A. limacine*) Mbs causing susceptibility of these Mbs to autoxidation (*38*).

The T_m value of bullet tuna Mb (75.0 °C) was lower than that of horse Mb (84.2 °C). This may be caused by the lower levels of α-helical contents (34.5% at 10 °C) compared with that of horse (55.3% at 10 °C). This T_m value was the lowest among those of all the scombridae fish Mbs examined in the previous report (*26*). Although the amino acid sequence identity between bullet tuna and skipjack Mbs was high (91.8%), the T_m value of skipjack Mb (79.9 °C) was the highest among all the scombridae fish Mbs (*26*). This may be caused partly by the substitutions of amino acid residues such as Pro13Ala, Gly62Ala, and Gln112His. Suzuki (*37*) claimed that lower hydrophobicity of heme pocket is related to lower stability of *Dolabella auricularia* Mb, estimated from the higher autoxidation rate. On the other hand, it was reported that some amino acids such as His12, Ala74, and Lys140 (positions in the final protein) of sperm whale Mb play key roles in its structural stability (*39*, *40*). Our previous study demonstrated that, despite the only two substitutions of amino acid residue between bigeye and bluefin tuna Mbs, their thermostability was clearly different (*26*). In bullet tuna Mb, several substitutions of amino acid residue were recognized among scombridae fish Mbs examined. Especially, substitution of Ala62Gly could result in perturbation of the structure of E segment forming a heme pocket, causing a slight decrease in its hydrophobicity and thus in the reduced thermostability.

The α-helical content of bullet tuna Mb was entirely lower than that of horse Mb and decreased dramatically in the pH range of 60−72.5 °C. It follows that the lower α-helical content was closely related to lower level of thermostability. Luo and Baldwin (*41*), using recombinant sperm whale Mb, demonstrated that the stability of α-helix against urea was increased only by a single or double mutation(s), e.g., Gly to Ala (G23A/G25A) in segment B. In our results, the decline of α-helical content and thermostability may be caused by such subtle substitution(s) of amino acid residues, causing a change of hydrophobicity of segment E.

In connection with this, bullet tuna cruise the surface (∼10 m) of coastal waters and around islands (*42*), unlike the other

tunas, which dive as deep as ~1000 m (*43*). Bullet tuna thus might not need such stable Mb as those of other tuna species which suffer from high hydraulic pressure during diving.

The isoelectric point (calculated value) of bullet tuna Mb (9.61) was higher compared with that of horse Mb (8.07). The molecular mass of bullet tuna Mb measured by MALDI-TOF MS (15407) was lower by 90 than the theoretical value calculated from deduced amino acid sequence (15497). The N-terminal residue could not be determined by Edman degradation (data not shown). These results suggest that N-terminus of bullet tuna Mb is posttranslationally modified. Moreover, mass spectra of the lysyl endoproteinase digest gave only a characteristic peak corresponding to the N-terminal acetylated octapeptide starting from the second Ala (*m/z* 921.4). It has been reported that the N-terminus of several scombridae fish Mbs is acetylated (*11*, *12*). Therefore, we came to a conclusion that the N-terminus of bullet tuna Mb is also acetylated, based on the detailed examination by PSD analysis (**Table 3**). Resultant actual values of product ion peaks detected corresponded to the theoretical values, suggesting that the N-terminal methionine was posttranslationally removed and the next alanine was acetylated. Although the role of N-terminal modification has not been well understood, it could provide a stability increase of the hemeprotein.

From all the above results, it was suggested that bullet tuna Mb has a characteristics similar to skipjack Mb, but the stability is slightly inferior to the latter. Bullet tuna could be utilized as a substitute for skipjack, though differences in flavor, texture, and postrigor deterioration of muscle would also exist. Further investigations are necessary to improve the commercial value of this fish species.

ABBREVIATIONS USED

Mb, myoglobin; DSC, differential scanning calorimetry; CD, circular dichroism; MALDI-TOF, matrix-assisted laser desorption/ionization-time-of-flight; MS, mass spectrometry; MS-MS, tandem mass spectrometry.

ACKNOWLEDGMENT

The authors thank Prof. S. Watabe, The University of Tokyo, for critically reading the manuscript.

LITERATURE CITED

(1) Kendrew, J. C.; Dickerson, R. E.; Strandberg, B. E.; Hart, R. G.; Davies, D. R.; Phillips, D. C.; Shore, V. C. Structure of myoglobin. *Nature* **1960**, *185*, 422−427.

(2) Phillips, S. E. V.; Schoenborn, B. P. Neutron diffraction reveals oxygen-histidine hydrogen bond in oxymyoglobin. *Nature* **1981**, *292*, 81−82.

(3) Birnbaum, G. I.; Evans, S. V.; Przybylska, M.; Rose, D. R. 1.70 Å resolution structure of myoglobin from yellowfin tuna. An example of a myoglobin lacking the D helix. *Acta Crystallogr*. **1994**, *D50*, 283−289.

(4) Fosmire, G. J.; Brown, W. D. Yellowfin tuna (*Thunnus albacares*) myoglobin: characterization and comparative stability. *Comp. Biochem. Physiol*. **1976**, *55B*, 293−299.

(5) Edmundson, A. B. Amino acid sequence of sperm whale myoglobin. *Nature* **1965**, *205*, 883−887.

(6) Dautrevaux, M.; Boulanger, Y.; Han, K.; Biserte, G. Covalent structure of horse myoglobin. *Eur. J. Biochem*. **1969**, *11*, 267−277.

(7) Han, K. K.; Tetaert, D.; Moschetto, Y.; Dautrevaux, M.; Kopeyan, C. The covalent structure of sheep-heart myoglobin. *Eur. J. Biochem*. **1972**, *27*, 585−592.

(8) Chauvet, J. P.; Acher, R. Isolation of coelacanth (*Latimeria chalumnae*) myoglobin. *FEBS Lett*. **1972**, *28*, 16−18.

(9) Colonna, G.; Irace, G.; Bismuto, E.; Servillo, L.; Balestrieri, C. Structual and functional aspects of the heart ventricle myoglobin of bluefin tuna. *Comp. Biochem. Physiol*. **1983**, *76A*, 481−485.

(10) Amano, H.; Hashimoto, K. Terminal structure of tuna and skipjack myoglobins. *Nippon Suisan Gakkaishi* **1976**, *42*, 577−581.

(11) Amano, H.; Hashimoto, K. N-terminal structure of fish myoglobin. *Nippon Suisan Gakkaishi* **1968**, *34*, 955−958.

(12) Rice, R. H.; Watts, D. A.; Brown, W. D. Sequences of the soluble tryptic peptides from myoglobin of yellowfin tuna (*Thunnus albacares*). *Comp. Biochem. Physiol*. **1979**, *62B*, 481−487.

(13) Chow, C. J.; Ochiai, Y.; Watabe, S.; Hashimoto, K. Effect of freezing and thawing on the discoloration of tuna meat. *Nippon Suisan Gakkaishi* **1988**, *54*, 639−648.

(14) Ochiai, Y.; Chow, C. J.; Watabe, S.; Hashimoto, K. Evaluation of tuna meat discoloration by Hunter color difference scale. *Nippon Suisan Gakkaishi* **1988**, *54*, 649−653.

(15) Aojula, H. S.; Wilson, M. T.; Morrison, I. E. G. Functional consequences of haem orientational disorder in sperm-whale and yellow-fin-tuna myoglobins. *Biochem. J*. **1987**, *243*, 205−210.

(16) Suzuki, N.; Hashimoto, K.; Matsuura, F. Studies on the color of skipjack meat. *Nippon Suisan Gakkaishi* **1973**, *39*, 35−41.

(17) Chow, C. J. Relationship between the stability and autoxidation of myoglobin. *J. Agric. Food Chem*. **1991**, *39*, 22−26.

(18) Chanthai, S.; Ogawa, M.; Tamiya, T.; Tsuchiya, T. Studies on thermal denaturation of fish myoglobins using differential scanning calorimetry, circular dichroism, and tryptophan fluorescence. *Fish. Sci*. **1996**, *62*, 927−932.

(19) Chanthai, S.; Ogawa, M.; Tamiya, T.; Tsuchiya, T. Studies on thermal denaturation of fish apomyoglobins using differential scanning calorimetry, circular dichroism, and tryptophan fluorescence. *Fish. Sci*. **1996**, *62*, 933−937.

(20) Kitahara, Y.; Matsuoka, A.; Kobayashi, N.; Shikama, K. Autoxidation of myoglobin from bigeye tuna fish (*Thunnus obesus*). *Biochim. Biophys. Acta* **1990**, *1038*, 23−28.

(21) Gutzke, D.; Trout, G. R. Temperature and pH dependence of the autoxidation rate of bovine, ovine, pig, and cervine oxymyoglobin isolated from three different muscles − *longissimus dorsi*, *gluteus medius*, and *biceps femoris*. *J. Agric. Food Chem*. **2002**, *50*, 2673−2678.

(22) Brown, W. D. The concentration of myoglobin and hemoglobin in tuna flesh. *J. Food Sci*. **1962**, *27*, 26−28.

(23) Matsuura, F.; Hashimoto, K. Chemical studies on the red muscle ("chiai") of fishes − II. Determinations of the content of hemoglobin, myoglobin and cytochrome *c* in the muscles of fishes. *Nippon Suisan Gakkaishi* **1954**, *20*, 308−312.

(24) Matsuura, F.; Hashimoto, K. Chemical studies on the red muscle ("chiai") of fishes − X. A new method for determination of myoglobin. *Nippon Suisan Gakkaishi* **1959**, *24*, 809−815.

(25) Funatsu, Y. The taste and flavor of fish sauce prepared by use of soy sauce koji. *Nippon Shokuhin Kagaku Kogaku Kaishi* **2002**, *49*, 1−11.

(26) Ueki, N.; Ochiai, Y. Primary structure and thermostability of bigeye tuna myoglobin in relation to those from other scombridae fish. *Fish. Sci*. **2004**, *70*, 875−884.

(27) Yang, J. T.; Wu, C. S. C.; Martinez, H. M. Calculation of protein concentration from circular dichroism. *Methods Enzymol*. **1986**, *130*, 208−269.

(28) Marcinek, D. J.; Bonaventura, J.; Wittenberg, J. B.; Block, B. A. Oxygen affinity and amino acid sequence of myoglobins from endothermic and ectothermic fish. *Am. J. Physiol. Regul. Integr. Comp. Physiol*. **2001**, *280*, R1123−1133.

(29) Akaboshi, E. Cloning and sequence analysis of porcine myoglobin cDNA. *Gene* **1985**, *40*, 137−140.

(30) Herrera, A. E.; Lehmann, H. Primary structure of human myoglobin. *Nature New Biol*. **1971**, *232*, 149−152.

(31) Springer, B. A.; Sligar, S. G. High-level expression of sperm whale myoglobin in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, 84, 8961−8965.

(32) Grove, T. J.; Sidell, B. D. Myoglobin deficiency in the hearts of phylogenetically diverse temperate-zone fish species. *Can. J. Zool*. **2002**, *80*, 893−901.
(33) Takeda, N.; Yamaguchi, K.; Hashimoto, K. Decolorization of dark muscle of mackerel and sardine. *Nippon Suisan Gakkaishi* **1980**, *46*, 1269−1272.
(34) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(35) Maiken, V.; Kroger, O.; Henrik, O.; Mogens, L. A. The effect of pH on the oxidation of bovine serum albumin by hypervalent myoglobin species. *Arch. Biochem. Biophys*. **2003**, *416*, 202−208.
(36) Chow, C. J.; Ochiai, Y.; Watabe, S., Hashimoto, K. Reduced stability and accelerated autoxidation of tuna myoglobin in association with freezing and thawing. *J. Agric. Food Chem*. **1989**, *37*, 1391−1395.
(37) Suzuki, T.; Muramatsu, R.; Kisamori, T.; Furukohri, T. Myoglobin of the shark *Galeus nipponensis*: identification of the exceptional amino acid replacement at the distal (E7) position and autoxidation of its oxy-form. *Zool. Sci*. **1988**, *5*, 69−76.
(38) Suzuki, T. Amino acid sequence of myoglobin from the mollusc *Dolabella auricularia*. *J. Biol. Chem*. **1986**, *261*, 3692−3699.
(39) Barrick, D.; Baldwin, R. L. The molten globule intermediate of apomyoglobin and the process of protein folding. *Protein Sci*. **1993**, *2*, 869−876.
(40) Scott, E. E.; Paster, E. V.; Olson, J. S. The stabilities of mammalian apomyoglobins vary over a 600-fold range and can be enhanced by comparative mutagenesis. *J. Biol. Chem*. **2000**, *275*, 27129−27136.
(41) Luo, Y.; Baldwin, R. L. How Ala→Gly mutations in different helices affect the stability of the apomyoglobin molten globule. *Biochemistry* **2001**, *40*, 5283−5289.
(42) FishBase: http://www.fishbase.org/Summary/SpeciesSummary.
(43) Gunn, J., Block, B. Advances in acoustic, archival, and satellite tagging of tunas. In *Tuna Physiology, Ecology, and Evolution*; Block, B. A., Stevens, E. D., Eds.; Academic Press: New York, 2002; pp 167−224.

Received for review February 3, 2005. Revised manuscript received April 6, 2005. Accepted April 19, 2005.

JF050261Y