Primary structure and thermostability of bigeye tuna myoglobin in relation to those of other scombridae fish

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ABSTRACT: In the present study, the cDNA encoding myoglobin (Mb) of bigeye tuna Thunnus obesus was cloned and its amino acid sequence deduced in order to investigate the relationship between the primary structure and thermostability of scombridae fish Mb. An open reading frame of bigeye tuna Mb cDNA contained 444 nucleotides encoding 147 amino acids. The primary structure of bigeye tuna Mb was highly conserved when compared with those of bluefin tuna and yellowfin tuna Mb, the sequence identity being 95.2–100.0%. It also showed relatively high identity (82.3–89.1%) with the counterparts of scombridae fish. Myoglobin was then isolated from the dark muscle of four scombridae fish including bigeye tuna. Differential scanning calorimetry and circular dichroism measurements on these Mb revealed that the thermostability of bigeye tuna Mb was lowest and that of skipjack Katsuwonus pelamis Mb highest among the scombridae fish Mb examined. The α-helical contents of scombridae fish Mb at 10°C were in the range of 39.8–44.8%, clearly lower than that of horse Mb (55.3%), suggesting instability of fish Mb. The melting temperatures of these Mb fell in the range of 75.7–79.9°C, lower than that of horse Mb (84.2°C). These results strongly suggest the instability of fish Mb.

KEY WORDS: bigeye tuna, cDNA cloning, circular dichroism, differential scanning calorimetry, myoglobin, primary structure, scombridae, thermostability.

INTRODUCTION

Myoglobin (Mb), one of the heme proteins, is found exclusively in muscle. Myoglobin was the first protein whose structure was solved by X-ray diffraction by Kendrew et al. on sperm whale Mb. The structure has recently been determined to a higher resolution than 1.2 Å by Vojtechovsky et al. These studies revealed that Mb is a relatively compact globular protein whose backbone structure consists of eight α-helical segments designated A–H. The heme resides in a hydrophobic ‘heme pocket’, and binds the imidazole group of proximal histidine directly and that of distal histidine through an oxygen coordinate binding. As the oxygen affinity level of Mb is higher than that of hemoglobin, Mb can store oxygen temporarily in muscle fiber and transfer it to an electron transfer system.

The crystal structure of yellowfin tuna Thunnus albacares Mb has been solved. Its structure is quite similar to that of sperm whale Mb, although the tuna Mb lacks a D-helix. This Mb is poor in lysine, histidine and glutamic acid, and rich in aspartic acid and alanine. In this connection, the contents of acidic amino acids are generally lower in scombridae fish Mb in comparison with those of other fish species. The N-terminal residue is valine or glycine in mammalian Mb, but is varied in fish Mb. It is acetylated in bluefin tuna Thunnus thynnus, skipjack Katsuwonus pelamis and yellowfin tuna Mb. The color of Mb changes depending on the degree of oxidation of heme, namely on the composition of deoxyMb, oxyMb and metMb, showing different visible absorption spectra. MetMb is produced by oxidation of oxyMb when the heme iron changes from a ferrous form to a ferric one. Although metMb is reduced through a reducing system in vivo, post-mortem accumulation of metMb proceeds because of inactivation of this system. This phenomenon greatly deteriorates the quality of meat, because accumulation of metMb turns the meat color to brown. Previous studies have shown that metMb is more susceptible to denaturation compared with the other forms, and the progression of color darkening of tuna meat through freezing and thawing is faster than in unfrozen meat.
Fish Mb is quite unstable compared with that of higher vertebrates, and tends to autoxidize and aggregate easily.\textsuperscript{4,11,17,18} Chow investigated the effects of pH on stability against guanidine hydrochloride and the autoxidation rate for scombridae fish Mb, and found that lower stability was closely related to a higher autoxidation rate.\textsuperscript{4,20–23} Other studies have also shown that differences in stability exist among those Mb observed with a thermal denaturation profile and autoxidation rate.\textsuperscript{4,20–23} Instability of fish Mb could be a commercially serious problem in scombridae fish that have a high amount of Mb.\textsuperscript{24–26} In the present study, in order to establish the relationship between the amino acid sequence and thermostability of scombridae fish Mb, attempts were made to clone cDNA encoding bigeye tuna \textit{Thunnus obesus} Mb, the sequence of which has not been determined so far despite the interest in the divergence of tuna Mb and the commercial importance of this species. The thermodynamic properties were then compared among Mb from several scombridae fish species, including bluefin tuna, yellowfin tuna and skipjack. Namely, Mb was isolated from the dark muscle of each of these species and their thermostability was compared by differential scanning calorimetry (DSC) and circular dichroism (CD) taking horse heart Mb as a control.

**MATERIALS AND METHODS**

**Materials**

Fresh specimens of bigeye tuna, bluefin tuna, yellowfin tuna and skipjack were purchased in Tokyo Metropolitan Central Wholesale Market (Tsukiji, Tokyo) and stored at −80°C until they were used for experiments. Horse Mb was purchased from Sigma Chemical (St Louis, MO, USA) as a control. The subsequent procedures were performed at 0–4°C unless otherwise stated.

**Isolation of RNA and synthesis of first-strand cDNA**

Total RNA was extracted from the ordinary (fast skeletal) muscle of bigeye 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 manufacturer’s protocols. First-strand cDNA was synthesized as follows. An aliquot of 5 μg of the total RNA was dissolved in 12 μL of water, incubated at 70°C for 10 min, and then quickly chilled on ice. The NotI oligo dT primer (5′-AACTGGAAGAT-TCGCGGCCGAGAA (T)\textsuperscript{18–3′}) was used at 50 μg/mL to initiate first-strand cDNA synthesis with 0.5 U SuperScript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA) in 50 mM Tris HCl (pH 8.3), containing 75 mM KCl, 3 mM MgCl\textsubscript{2}, 500 μM dNTP and 10 mM dithiothreitol. The reaction was carried out at 42°C for 1 h in a total volume of 20 μL, and then the enzyme was heat-inactivated at 70°C for 15 min. RNase H (10 μg) (Invitrogen Corp.) was added to degrade the RNA template.

**cDNA cloning of bigeye tuna myoglobin**

Nucleotide sequences of primers used for polymerase chain reaction (PCR) are shown in Table 1. The location and combination of primers are indicated in Fig. 1. Primers MF1 and MR1 were designed with reference to the highly conserved regions of the nucleotide sequences of Mb cDNA (corresponding to Ala9-Pro13 and Arg126-Ile131) from bluefin tuna, albacore, yellowfin tuna and Antarctic Nototheniidae fish (\textit{Champsocephalus gunnari} and \textit{Cryordraco antarcticus}) (these sequence data have been submitted to the GenBank database under accession numbers AF291831, AF291832, AF291838, U71054 and U71056, respectively).\textsuperscript{27} The PCR was initiated by adding 1 μg of first-strand cDNA as a template to 20 μL of the reaction mixture containing 2 μL of 10 × Ex \textit{Taq} buffer (Takara, Otsu, Japan), 200 μM dNTP mixture, 10 pmol of primer MF1, 10 pmol of primer MR1 and 0.1 U of Ex \textit{Taq} DNA polymerase (Takara). The PCR consisted of initiating a denaturation step at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The final extension step was performed at 72°C for 5 min. After PCR products were subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), nucleotides were sequenced by an ABI PRISM 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA) using BigDye Terminator Cycle Sequencing Kit Version 3 (Applied Biosystems).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>MF1</td>
<td>5′-TGAAGTGT (C/T) TGGGGTCCAGGT-3′</td>
</tr>
<tr>
<td>MR1</td>
<td>5′-GAT (A/G) (C/G) CCACTACGTTCCTCA-3′</td>
</tr>
<tr>
<td>M5R1</td>
<td>5′-CTTTTGGCCAGTGTTT-3′</td>
</tr>
<tr>
<td>M5R2</td>
<td>5′-TTCCAGCACTTCCTCCAGTT-3′</td>
</tr>
<tr>
<td>M5R3</td>
<td>5′-AACAGCTTCTGGTCTCAG-3′</td>
</tr>
<tr>
<td>M3R1</td>
<td>5′-GGTGAAGAAACTTGAGGGC-3′</td>
</tr>
<tr>
<td>M3R2</td>
<td>5′-TGCAATCTCIAAAACCACCTG-3′</td>
</tr>
</tbody>
</table>
Primers M5R1, M5R2, M5R3, M3R1 and M3R2 for rapid amplification of cDNA ends (RACE) were designed based on the internal sequence of bigeye tuna Mb cDNA obtained (Fig. 1). In 3’ RACE, PCR was performed with the first-strand cDNA as a template and the primers M3R1, M3R2 and an abridged universal amplification primer (5’-GGC-CAGCGCTGACTAGTAC-3’). The PCR was carried out at 94°C for 3 min, followed by 35 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. 5’ RACE was performed using the 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 M5R1. Subsequently, PCR was carried out with primers M5R2 and M5R3 at 94°C for 3 min, followed by 30 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.

Subcloning and nucleotide sequencing of both strands were performed according to the above methods. Based on the nucleotide sequences of bigeye tuna Mb cDNA obtained, the amino acid sequence was deduced. A phylogenetic tree was drawn based on the nucleotide sequences of the Mb cDNA of bigeye tuna and other species by the neighbor-joining method, and the sequences of mammalian Mb were used as an out-group to root the tree.27-31 Alignment of the deduced amino acid sequences was performed using the Clustal W program (EBI, Cambridge, UK).

**Purification of myoglobin**

The dark (slow skeletal) muscle was excised, minced in a mortar and extracted with two volumes of distilled water for 20 min. After centrifugation at 7500 x g for 15 min, the supernatant was filtered through filter paper (No.2, Advantec Toyo, Tokyo, Japan). 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 x 100 cm; Amersham Biosciences, Piscataway, NJ, USA) equilibrated with 50 mM Tris HCl (pH 8.0) at a flow rate of 60 mL/h. The elution pattern was monitored by absorbance at 280 and 540 nm. The Mb fraction was further purified by ion-exchange chromatography using a TOYOPEARL CM-650M column (15 mm x 30 cm; TOSOH Corp., Tokyo, Japan) equilibrated with 1.5 mM Tris phosphate (pH 7.0). The Mb was eluted with a linear gradient using 500 mL each of 1.5 mM and 15.0 mM Tris phosphate (pH 7.0) at a flow rate of 45 mL/h. The elution was monitored as described above. The purity of the Mb was checked by sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis (17.5% gel).

**Differential scanning calorimetry**

Differential scanning calorimetry was performed with a MicroCal VP-DSC differential scanning microcalorimeter (MicroCal Inc., Northampton, MA, USA).22,32 Protein concentration was adjusted to 1.0 mg/mL in 10 mM sodium phosphate (pH 7.0) containing 0.15 M KCl. The DSC scan was carried out at a rate of 60°C/h in the temperature range of 10–100°C. Differential scanning calorimetry data were analyzed using a software package (Origin) developed by MicroCal Inc. The heat capacity data were fitted by using non-linear least-squares, initially assuming that $\Delta H_{cal}/\Delta H_{th} = 1$, where $\Delta H_{cal}$ and $\Delta H_{th}$ are the calorimetric and van’t Hoff enthalpy, respectively. When data did not fit satisfactorily, heat capacity curves were subsequently fitted by allowing $\Delta H_{cal}$ and $\Delta H_{th}$ to float. After minimization by appropriate computer programs, the values for the thermal transition, including the midpoint of melting temperature (Tm) and the molar excess heat capacity, were obtained.

**Circular dichroism**

Circular dichroism measurement was carried out with a spectropolarimeter (JASCO J-700 W; JASCO
Corp., Tokyo, Japan) using a 0.2-mm water-jacketed cylindrical cell in the temperature range of 10.0–85.0°C with an increment of 5.0°C (10–60°C) or 2.5°C (60–85°C). The wavelength for measurement was in the range of 240–195 nm. The decreasing rate derivatives were calculated from the increment of the mean residue ellipticity at 222 nm per unit change of temperature. The determination of α-helical content was performed according to Yang et al. with 200 µg/mL of Mb in the same buffer used for DSC.34

Other analytical methods

Protein concentration was determined by BCA Protein Assay Kit (Pierce, Rockford, IL, USA) using horse Mb as a standard, according to the manufacturer’s protocols.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis was performed by the method of Laemmli using 17.5% gel.35 The standard molecular weight marker kit used was SDS-7 (Sigma Chemical).

Molecular weight was measured by MALDI-TOF mass spectrometry using a Voyager DE-STR (Applied Biosystems).

RESULTS

cDNA cloning of bigeye tuna myoglobin

Bigeye tuna Mb cDNA was prepared from ordinary muscle, because it is very difficult to prepare total RNA of high quality from dark muscle. An open reading frame of bigeye tuna Mb cDNA contained 444 nucleotides, encoding 147 amino acids (Fig. 2). The coding region was preceded by a 5’ non-coding region of 81 base pairs (bp) and was followed by a 3’ non-coding region of 267 bp, and there was no sign of the presence of isoform. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number AB104433. The isoelectric point calculated by using the deduced amino acid sequence was 9.81, and the molecular mass was 15 628 Da, as calculated from amino acid composition, and 15 540 Da, as determined by mass spectrometry.

The primary structure of bigeye tuna Mb was found to be highly conserved when compared with those of bluefin and yellowfin tuna (the amino acid sequence identities were 100.0 and 98.6%, respectively; Fig. 3; Table 2). Incidentally, the nucleotide sequences of the open reading frame were 99.8 and 99.5% identical, respectively. In these tuna Mb, Pro13 in segment A was considered to make a kink in the helical structure. Atlantic blue marlin Makaira nigricans, skipjack and chub mackerel Scomber japonicus Mb, however, showed less homology: the amino acid sequence identities were 85.7, 85.0 and 82.3%, respectively, although several conservative regions – for instance, Thr36–Ile46 and Ala 59–Leu68 – were recognized. Conversely, the amino acid identities of bigeye tuna Mb were at high levels (76.1–80.2%) when compared with those of Nototheniidae fish (humped rockcod Gobionotothen gibeliofrons, black rockcod Notothenia coriiceps and sea raven Hemitripterus americanus) and those with mammals (horse Equus caballus, Norwegian rat Rattus norvegicus, pig Sus scrofa and sperm whale Physeter macrocephalus) and a mollusk (sea hare Aplysia juliana) were at much lower levels (42.9–45.6 and 19.0%, respectively).

Fig. 2 Nucleotide and deduced amino acid sequences of bigeye tuna myoglobin cDNA. The initiation codon is indicated in bold, and the termination codon is indicated by an asterisk. Amino acid numbers are indicated in bold and italics on the right. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number AB104433.
Distal and proximal histidine residues (His60 and His89, respectively, in Fig. 3) that bind heme were conserved throughout all the scombridae fish and mammalian (pig and sperm whale) Mb. The identities of amino acid sequence differed depending on the region of the Mb molecule (Table 3).

Table 3  Identities of the amino acid sequence in the α-helical segments and intersegmental regions between bigeye tuna and scombridae myoglobin

<table>
<thead>
<tr>
<th>Region</th>
<th>Identity (%)</th>
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<tbody>
<tr>
<td>A</td>
<td>9/14 (64.3)</td>
</tr>
<tr>
<td>B</td>
<td>8/15 (53.3)</td>
</tr>
<tr>
<td>C</td>
<td>5/5 (100.0)</td>
</tr>
<tr>
<td>C-E</td>
<td>11/17 (64.7)</td>
</tr>
<tr>
<td>E</td>
<td>15/18 (83.3)</td>
</tr>
<tr>
<td>E-F</td>
<td>5/7 (71.4)</td>
</tr>
<tr>
<td>F</td>
<td>6/9 (66.6)</td>
</tr>
<tr>
<td>F-G</td>
<td>5/7 (71.4)</td>
</tr>
<tr>
<td>G</td>
<td>9/16 (56.3)</td>
</tr>
<tr>
<td>G-H</td>
<td>6/8 (75.0)</td>
</tr>
<tr>
<td>H</td>
<td>14/20 (70.0)</td>
</tr>
</tbody>
</table>

The numbers for intersegments A-B and B-C were not included because both contained only one residue.

**Table 2  Identities of the amino acid sequence of bigeye tuna myoglobin with those of other species**

<table>
<thead>
<tr>
<th>Species†</th>
<th>Identity (%)</th>
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<tbody>
<tr>
<td>Bluefin tuna</td>
<td>100.0</td>
</tr>
<tr>
<td>Yellowfin tuna</td>
<td>98.6</td>
</tr>
<tr>
<td>Albacore</td>
<td>95.2</td>
</tr>
<tr>
<td>Pacific bonito</td>
<td>89.1</td>
</tr>
<tr>
<td>Atlantic blue marlin</td>
<td>85.7</td>
</tr>
<tr>
<td>Skipjack</td>
<td>85.0</td>
</tr>
<tr>
<td>Chub mackerel</td>
<td>82.3</td>
</tr>
<tr>
<td>Humped rockcod</td>
<td>80.2</td>
</tr>
<tr>
<td>Black rockcod</td>
<td>78.9</td>
</tr>
<tr>
<td>Sea raven</td>
<td>76.1</td>
</tr>
<tr>
<td>Horse</td>
<td>45.6</td>
</tr>
<tr>
<td>Human</td>
<td>44.9</td>
</tr>
<tr>
<td>Pig</td>
<td>43.5</td>
</tr>
<tr>
<td>Norway rat</td>
<td>43.5</td>
</tr>
<tr>
<td>Sperm whale</td>
<td>42.9</td>
</tr>
<tr>
<td>Sea hare</td>
<td>19.0</td>
</tr>
</tbody>
</table>

†These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under the following accession numbers: bluefin tuna, AF291831; yellowfin tuna, AF291832; Albacore, AF291833; Pacific bonito, AF291834; Atlantic blue marlin, AF291835; skipjack, AF291837; chub mackerel, AF291835; humped rockcod, U71057; black rockcod, U71058; sea raven, AY029587; horse, P01288; human, NM_005368; pig, M14433; Norway rat, AF197916; sperm whale, J03566; sea hare, AB003277.

identities of segment E and intersegment G-H were relatively high among scombridae fish Mb. In contrast, the sequence identities of segments B and G were low (53.3 and 56.3%, respectively).

Skipjack Mb was one residue shorter than tuna Mb. This deletion was in the intersegment C-E. Skipjack Mb showed another specific feature, which is that it has four more alanine residues (Ala13, 27, 105 and 107) in the α-helical segments compared with tuna Mb. In particular, the replacement of Pro13 by Ala13 in segment A is considered to favor stabilization of this segment, because proline perturbs the α-helix.
of Atlantic blue marlin, those of scombridae fish formed a cluster and were found to be closely related to each other. In particular, bigeye tuna Mb was located close to the Mb of bluefin tuna, yellowfin tuna and albacore. Nototheniidae fish Mb formed another cluster.

**Differential scanning calorimetry measurement**

As shown in Fig. 5, Mb was purified from the dark muscle of four scombridae species (bigeye tuna, bluefin tuna, yellowfin tuna and skipjack). Their thermostability was compared by DSC measurement, taking as a standard horse heart Mb whose amino acid sequence was already known. A single distinct endothermic peak was observed in the range of 75–85°C for all the Mb examined (Fig. 6). A DSC scan was performed twice sequentially for each sample, but in the second run, no endothermic peak was observed (data not shown). The $T_m$ values of scombridae fish Mb (75.7–79.9°C) were clearly lower than that of horse Mb (84.2°C). Notably, that of bigeye tuna Mb (75.7°C) was the lowest. Of all the scombridae fish Mb examined, the highest value of $T_m$ (79.9°C) occurred among skipjack Mb. The $T_m$ values of bluefin (78.6°C) and yellowfin (78.2°C) tuna Mb were very close to each other.

**Circular dichroism measurement**

Ellipticity values at 222 nm ($[\theta]_{222}$) increased as the temperature was raised (Fig. 7). The values at 10°C were in a range from −17 900 to −15 900 for scombridae fish Mb. That is, the $\alpha$-helical contents were in a range from 39.8 to 44.8%. These values were lower than those of horse Mb (55.3%). Among fish Mb, the lowest mean residue ellipticity occurred in skipjack (−17 900), and the values for the other three species fell in a narrow range (bigeye tuna, −15 900; bluefin tuna, −16 000; yellowfin tuna, −15 900). The increment of the mean residue ellipticity became steeper. The decreasing rate derivative of bigeye tuna Mb (0.14) was high compared with that of horse Mb (0.06) in the temperature range of 60–70°C. Skipjack Mb gave intermediate values between tuna and horse Mb concerning the temperature dependence of ellipticity change. It was also confirmed that skipjack Mb was more stable than tuna Mb.
DISCUSSION

As a result of cDNA cloning of bigeye tuna Mb, its primary structure was found to resemble those of other tuna Mb. The amino acid sequence was identical for bigeye and bluefin tuna Mb, and only two substitutions (in segment B and intersegment C–E) were found between those two species and yellowfin tuna Mb. The nucleotide sequence identity of the coding region was 99.5% between bigeye tuna and yellowfin tuna. Among the scombridae species examined, amino acid sequence identities were lowest between bigeye tuna and chub mackerel. Conversely, phylogenetic analysis showed that Atlantic blue marlin was most remotely related. The sequence identities were quite low with mammalian and mollusk Mb (43.5–45.6 and 19.0%, respectively). Both the distal and proximal histidine residues (His60 and His89) in tuna Mb examined in the present study were conserved without exception. Distal histidine residues are known to be replaced by glutamine or valine in shark Galeus nipponensis and some alysidae (Dolabella auricularia, Aplysia kurodai, A. juliana and A. limacina) Mb.36,37

The isoelectric point (calculated value) of bigeye tuna Mb (9.81) was high compared with that of horse Mb (8.07). Molecular mass measured by MALDI-TOF mass spectrometry (15 540 Da) was lower by 88 Da than the calculated value from the deduced amino acid sequence (15 628 Da). In case of acetylation of the N-terminus, the increment of molecular mass is 42. In fact, the N-terminus residue of bigeye tuna Mb could not be determined by

**Fig. 5** Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis patterns of purified myoglobin (Mb). M, markers (SDS-7); 1, bigeye tuna; 2, bluefin tuna; 3, yellowfin tuna; 4, skipjack; 5, horse. Five micrograms of Mb was applied to each lane (17.5% gel).

**Fig. 6** Differential scanning calorimetry patterns of myoglobin from (a) bigeye tuna, (b) bluefin tuna, (c) yellowfin tuna, (d) skipjack and (e) horse. Differential scanning calorimetry was performed in 10 mM sodium phosphate (pH 7.0) containing 0.15 M KCl. The heating rate was 60°C/h in a temperature range of 10–100°C. The protein concentration was 1.0 mg/mL.
Edman degradation (data not shown). By taking this modification into account, the difference between the calculated and measured values could be 130. This value can be accounted for by assuming that the N-terminal methionine is removed after translation, and the next alanine is acetylated.

In the present experiments, cDNA was cloned from the ordinary muscle of bigeye tuna, whereas Mb was isolated from the dark muscle, because the Mb concentration of dark muscle is much higher than that of ordinary muscle. As described above, no isoform of Mb was recognized. In addition, no report is available so far about an Mb isoform. Thus, Mb from the ordinary and dark muscles is considered to be the same. This was further demonstrated by 2-D gel electrophoresis (data not shown).

It has been reported that, in DSC measurement, yellowfin tuna and bonito Mb give two peaks at approximately 60 and 65°C in 10 mM phosphate (pH 7.0) containing 0.15 M KCl. In the present study, however, only one peak was observed for all the species examined. The Tm values of scombridae fish Mb were clearly lower than that of horse Mb, suggesting that the thermostability of scombridae fish Mb is lower than that of a mammalian counterpart. This could be caused by the lower levels of α-helical contents (39.8–44.8%) in scombridae fish Mb. These values are close to those obtained by Fosmire and Brown (42–46%). Chan-thai et al. reported a relation between Tm values and the α-helical contents of Mb. Moreover, a clear decline in the α-helical contents of tuna Mb compared with that of horse Mb was considered to reflect their lower levels of thermostability. These results suggest that scombridae fish Mb is susceptible to irreversible denaturation, especially in a temperature range higher than Tm.

Suzuki claimed that lower hydrophobicity of the heme pocket is related to lower stability of D. auricularia Mb, estimated from a higher autoxidation rate. In contrast, it has been reported that some amino acids, such as His12, Ala74 and Lys140 (sequence in the final protein) of sperm whale Mb play key roles in its structural stability. These residues in whale Mb are replaced by Lys9, Glu70 and Ala134 in tuna Mb, respectively. In porcine Mb, these residues are Asn, Ala and Asn, respectively. Differences in stability between pig and tuna Mb could be partly caused by these substitutions on α-helical segments A, E, and H.

The lengths of α-helical segments are comparable between whale and tuna Mb, but tuna Mb has shorter segments F and H. These differences would also affect the stability of Mb. In connection with this, molten globule studies on apomyoglobin by Luo and Baldwin demonstrated that the stability of α-helix against urea was changed only by a single mutation or double mutations. The stability of segments A, G and H in a molten globule intermediate state was found to be important for unfolding. Differences in thermostability among Mb could be caused by those substitutions in hydrophobicity around the heme pocket and in α-helical contents as a result of a few substitutions of amino acid residues at critical positions.

Chow reported the differences in autoxidation rate and free energy for denaturation by guanidine hydrochloride among tuna Mb. Accordingly, the order of stability of Mb was bluefin tuna > yellowfin tuna > bigeye tuna. In the present study, differences in thermostability were also observed among scombridae fish Mb, and the Tm values in DSC analyses coincided with the above order of stability.

Interestingly, despite an identical deduced primary structure for bigeye and bluefin tuna Mb, they exhibited a slight but clear difference in thermostability, suggesting their tertiary structures or post-translational modifications are different. The N-terminal residues of fish Mb are known to be mostly acetylated. It is likely that some other part of Mb is also modified, affecting the stability...
of this hemoprotein. Studies to elucidate such post-translational modification in fish Mb are now in progress.

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