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# Effects of point mutations on the structural stability of tuna myoglobins

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ABSTRACT

Structural stabilities of myoglobin (Mb) from several tuna fish species significantly differ from each other, although the amino acid sequence identities are very high (>95%), suggesting that limited number of substitutions greatly affect the stability of Mb. To address this hypothesis, attempts were made to elaborate recombinant tuna Mbs with point mutations on the different residues among fish Mbs. The expression plasmid constructs were based on bigeye tuna Mb cDNA sequence, and the recombinant proteins were expressed as GST-fusion proteins in *Escherichia coli.* After removal of the GST segment and affinity purification, the stability of five Mb mutants, namely, A49G, T91K, K92Q, V108A, and H112Q, together with the wild type (WT) were measured, taking temperature dependency of α-helical content and denaturant (urea and guanidine-HCl) concentration dependency of Soret band absorbance as parameters. As a result, the mutant H112Q showed much higher stability than WT, while the structures of K92Q, T91K and A49G mutants were destabilized. No essential change in helical content was observed for V108A, but the mutant was found to be destabilized easier by the denaturants. These findings suggested that the highly conserved residues among tuna species are responsible for their stability of Mbs, but a few non-conserved residues dramatically give rise to the differences in stability of Mbs among species.

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## 1. Introduction

Myoglobin (Mb) is involved in oxygen storage in muscle by virtue of a coordinate bond between imidazole group of distal histidine and oxygen (Phillips and Schoenborn, 1981). In addition, new functions have recently been annotated to this hemoprotein such as scavenging nitrogen oxide and reactive $O_2$ species (Brunori, 2001; Wunderlich et al., 2003; Cossins and Berenbrink, 2008). Mb, molecular weight of which is generally ~17,000, consists of eight α-helical segments A through H from the N terminus (Kendrew et al., 1960; Vojtechovsky et al., 1999), though crystallographic analysis showed that fish (yellowfin tuna *Thunnus albacares*) Mb lacks D helix (Birnbaum et al., 1994). The heme is located in the hydrophobic "heme pocket", and binds imidazole group of proximal histidine directly and through the coordinate bond. As far as fishes are concerned, Mb is widely distributed in muscle, particularly in slow (dark) and cardiac muscles (Block and Stevens, 2001). Only recently, Fraser et al. (2006) reported that under hypoxia, expression of Mb is induced in several tissues other than muscles in carp *Cyprinus carpio*.

Stabilities of apo Mb, molten globule intermediates, heme binding, and intramolecular electron transfer have been investigated extensively by site-directed mutagenesis techniques (Tcherkasskaya et al., 2000; Hirota et al., 2005; Bourgeois et al., 2006). In connection with this, substitution of porcine Mb Ser92 with Leu or Ala promoted dissociation of heme (Smerdon et al., 1993). The mutations of distal His64, Val 68, and Arg45 also resulted in heme dissociation (Hargrove et al., 1994). The apo-form intermediate of sperm whale Mb was greatly affected by the mutation of Gln8 or Glu109 (Luo et al., 1997). Stability of sperm whale apo Mb was reinforced by replacement Pro in helix F with Ala, as demonstrated by its resistance against proteolysis (Picotti et al., 2004). On the other hand, stability of sea hare Mb intermediate was reduced by replacement of Trp130 with Tyr (the residue corresponding to Trp131 of sperm whale Mb) which is important for the packing of helices A, G and H observed at the initial state of folding (Musto et al., 2004). These findings support the view that a limited number of amino acid residues can be involved in the formation of hydrophobic heme pocket and stabilization of Mb.

The species of Tribe Thunnini (i.e., tunas and skipjack) have a large amount (~50 mg/g) of Mb in their skeletal muscles (not only in slow muscle but also in fast muscle) facilitating their sustained high speed locomotion activity. In our previous studies (Ueki and Ochiai, 2004, 2005, 2006; Ueki et al., 2005), it has been demonstrated that structural stabilities of scombridae fish Mbs clearly differ between the species examined, although the amino acid sequence identities were in the range of 91–99%. Clear difference in stability was observed between bigeye tuna *Thunnus obesus* and yellowfin tuna Mbs by taking several parameters (autooxidation rate, thermostability), though there are only two amino acid substitutions between them (Chow, 1991; Ueki and Ochiai, 2004). Among the scombroid Mbs studied, skipjack tuna *Katsuwonus pelamis* Mb was found to be the most thermostable in contrast to bullet tuna *Auxis rochei* Mb of the

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**Table 1**
Sequences of primers used for PCR.

| Mutants | Sequence |
|---|---|
| A49G | 5′-CTATGTCACCCTGGGCGATG-3′ |
| T91K | 5′-CTTGTGCTTCTTGGCATGGC-3′ |
| K92Q | 5′-GAATCTTGTGCTGAGTGGCATG-3′ |
| V108A | 5′-CATGACCTTCGCAAGGACCTC-3′ |
| H112Q | 5′-GCCTTCTCCTGCATGACCTTC-3′ |

lowest stability (Ueki and Ochiai, 2005). In this connection, Madden et al. (2004) concluded that it was difficult to identify the residues responsible for the structural flexibility of Mbs from four fish species, because the residue differences are distributed throughout the molecule. In this sense, scombroid fish Mbs are the ideal targets for pinpointing the residues controlling the stability of Mbs by following the stability change of recombinant proteins with point mutations.

Stability of Mb has been measured by various techniques. Bismuto et al. (2001) measured the extent of structural perturbation of tuna apo Mb by fluorescence correlation spectroscopy and found that ANS binding rate (in nanosecond order) of tuna apo Mb mainly to the hydrophobic heme pocket was much faster than the horse counterpart, suggesting that the former is in the open configuration or is more flexible. By the calculation of the molecular surface area, tuna apo Mb was found to have a 15% smaller cavity compared to its horse counterpart. We have used the decay of Soret band absorption for monitoring the structural perturbation of Mb (Ueki and Ochiai, 2006) in addition to circular dichroism (CD) and differential scanning calorimetry (DSC) measurements (Ueki and Ochiai, 2004; Ueki et al., 2005).

In the present study, the amino acid residue(s) that is important in the structural stability of tuna Mbs are to be elucidated using five Mb mutants, namely, A49G, T91K, K92Q, V108A, and H112Q, which were designed based on the sequence diversity of Mbs with the recombinant bigeye tuna Mb as a wild type. The effects of these mutations were examined taking as parameters, temperature dependency of α-helical contents and denaturant concentration dependency of Soret band absorbance.

## 2. Materials and methods

### 2.1. Preparation of mutant Mb

Site-directed mutagenesis was performed using polymerase chain reaction (PCR) as reported previously using the mutagenic primers (Table 1) (Ueki and Ochiai, 2005). The pGEX-2T plasmid containing bigeye tuna *T. obesus* wild type (WT) Mb cDNA was constructed as reported previously (Ueki and Ochiai, 2004). The purified PCR products were used as 5′ mutagenic primers for the second PCR. The final PCR products were digested with *Bam*HI and *Sma*I, subcloned into a pGEX-2T vector, and were sequenced with an ABI PRISM 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA) using BigDye Terminator Cycle Sequencing Kit Version 3 (Applied Biosystems).

Five mutants A49G, T91K, K92Q, V108A and H112Q were prepared in the present study. The mutation sites were determined due to the following reasons. Lys91, Gln92 and Ala108 are the substitutions present specifically in skipjack tuna Mb whose stability was the highest among all the scombridae fish Mbs examined (Ueki and Ochiai, 2004). Gly49 and Gln112 are the specific mutations in bullet tuna Mb whose stability was the lowest among all the scombridae fish Mbs examined (Ueki et al., 2005). These residues were replaced so that skipjack tuna Mb residues are turned into those of bullet tuna and vice versa.

The host strain *E. coli* BL21 (DE3) pLysS (Novagen, San Diego, California, USA) was transformed by the pGEX-2T expression vector (GE Healthcare, Little Chalfont, Buckinghamshire, England). Subsequently, hemin (Sigma-Aldrich, St. Louis, MO, USA) was added to the final concentration of 20 mg/L, and the expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 6 h. After spinning down the bacteria at 3000 ×*g* for 15 min at 4 °C, they were subjected to sonication in 50 mM Tris–HCl (pH 8.0) containing 150 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol (DTT) on ice. After addition of 10% (w/v) Triton X-100, the sonicate was subjected to ultracentrifugation at 12000 ×*g* for 30 min at 4 °C, and the supernatant was applied to further purification. The recombinant Mb was purified with a GSTrap column (GE Healthcare). The GST portion was cut from the fusion protein with thrombin, followed by the same affinity purification to separate Mb portion from GST. Thrombin was subsequently removed with a Benzamidine Sepharose 6B column (GE Healthcare). The purified recombinant Mbs were further incubated with two-fold molar excess of hemin to complete their folding.

### 2.2. α-helical contents

The α-helical contents in the recombinant Mbs were measured by CD with a J-700W spectropolarimeter (JASCO, Tokyo, Japan) using a water-jacketed cylindrical cell (0.2 mm). Measurement was carried out in the range of 10–87.5 °C from 240 through 195 nm, with 0.2 mg/mL of Mb in 10 mM Na-phosphate (pH 7.0) containing 150 mM KCl.

### 2.3. Unfolding profiles in the presence of denaturants

Unfolding of Mb in the presence of denaturants was estimated by the decrease in Soret band absorbance at 405 nm (Puett, 1973). Concentrations of the two denaturants, urea and guanidine hydrochloride (GdnHCl) were changed in the ranges of 0–6.5 M and 0–2.5 M, respectively, by adding the concentrate solutions to Mb in 10 mM Na-phosphate (pH 7.0) containing 150 mM KCl. Protein concentration was finally adjusted in the range of 0.1–0.2 mg/mL. The measurements were carried out three times for each sample.

Free energy for denaturation ($\Delta G_D$) was calculated from the following equations,

$$K_D = (E_N - E_{obs}) / (E_{obs} - E_D)$$

where $K_D$ is the equilibrium constant of denaturation, $E_N$ and $E_D$ are the molar extinction coefficients of protein in the native and denatured states, respectively, and $E_{obs}$ is the observed molar extinction coefficient between the native and denatured states.

$$\Delta G_D = -RT \ln K_D$$

where $R$ is the gas constant (1.987 cal deg$^{-1}$ mol$^{-1}$) and $T$ is the absolute temperature. The free energy stability of protein in the absence of denaturant was calculated by the equation,

$$\Delta G^0_{H_2O} = \Delta G_D + mC$$

where $m$ is the slope of regression line, and $C$ is the molar concentration of denaturant. The denaturant concentration of transition midpoint (*Cm*) was used as a parameter of structural perturbation.

### 2.4. Other experimental methods

Tertiary structure of Mb was depicted using a program MolFeat v.3.6 based on the data of blackfin tuna *Thunnus atlanticus* Mb (PDB 2NRT) (Schreiter et al., 2007). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 17.5% gel. Protein concentration was determined by BCA Protein Assay Kit (Pierce, Rockford, IL, USA), using horse Mb (Sigma-Aldrich) as a standard.

Deduced amino acid sequences were aligned by ClustalW (Thompson et al., 1994). The referred sequences are as follows: bigeye tuna *T. obesus* Mb (DDBJ/EMBL/GenBank accession number

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