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Temperature-dependent Structural Perturbation of Tuna Myoglobin

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***Abstract*—**To unveil the mechanism of fast autooxidation of fish myoglobins, the effect of temperature on the structural change of tuna myoglobin was investigated. Purified myoglobin was subjected to preincubation at 5, 20, 50 and 40°C. Overall helical structural decay through thermal treatment up to 95°C was monitored by circular dichroism spectrometry, while the structural changes around the heme pocket was measured by ultraviolet/visible absorption spectrophotometry. As a result, no essential structural change of myoglobin was observed under 30°C, roughly equivalent to their body temperature, but the structure was clearly damaged at 40°C. The Soret band absorption hardly differed irrespective of preincubation temperature, suggesting that the structure around the heme pocket was not perturbed even after thermal treatment.

***Keywords*—**denaturation, myoglobin, stability, tuna.

I. Introduction

MYOGLOBIN is a kind of hemoprotein, present in a large amount in skeletal and cardiac muscles of vertebrates. Myoglobin generally takes part in cellular respiration as an oxygen reservoir by forming a coordinate bonding between an imidazole group of distal His residue and oxygen [1]. Myoglobin molecule consists of seven to eight α-helical segments (designated A through H from the N terminus side) and a heme portion located in a hydrophobic heme pocket [2,3].

As far as fish myoglobins are concerned, they are especially distributed in muscles, particularly in slow skeletal (dark) and cardiac muscles [4]. Tunas generally accumulate a large amount of myoglobin in their skeletal muscles, not only in dark muscle but also in fast skeletal (white) muscle. Structural stabilities of tuna myoglobins differ clearly among the species, even though their amino acid sequence identity is in the range of 91-99% [5,6]. Several point mutation studies have been reported to specify the amino acid residues which are involved in the structural stability of myoglobins [7,8]. The studies carried by our research group have succeeded in identifying amino acid residues involved in structural stabilization and further indicated that tuna myoglobins are the ideal model proteins to establish the structure-stability relationship of this protein [9,10]. It is, however, difficult to pinpoint the residues involved in structural instability of myoglobins from different species, because the residue differences were found to be present along the entire molecule [11]. And the relationship between thermal stability and autooxidation rate of myoglobins remains unexplained.

Fish myoglobins are more unstable than the mammalian counterpart when autooxidation rate and aggregation profiles were compared [5,12,13]. This tendency is closely related to the fast discoloration rate of tuna meat [4,14,15]. It still remains unclear why fish myoglobins are oxidized so fast compared to mammalian myoglobins and how their structures are perturbed under denaturing conditions. A few reports are available on the thermal denaturation pattern of tuna myoglobin [5,6,16]. However, information on the stability of native tuna myoglobins remains in its infancy. Studies from this viewpoint are necessary to further understand denaturation mechanism of myoglobin and prevent its denaturation. The stability of myoglobin is considered to be closely related to that of helical region and heme pocket. In the present study, myoglobin was isolated from the fast skeletal muscle of tuna, and the temperature-dependent structural change of myoglobin was examined.

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II. Materials and Methods

A. Materials

Slow skeletal muscle was excised from a live specimen of cultured bluefin tuna *Thunnus orientalis* (body weight 46.7 kg, fork length 131 cm). The excised muscle was stored at -80 °C until used. All the chemicals used were of reagent grade.

B. Purification of myoglobin

Myoglobin was isolated from the dark muscle as reported previously [17]. Briefly, water extract of minced dark muscle was subjected to ammonium sulfate fractionation in the range of 70 - 90% saturation. The fraction was applied to a HiLoad 16/60 Superdex 200 gel filtration column (GE Healthcare) equilibrated with 0.5 mM Tris phosphate (pH 7.0), and the proteins were eluted with the same buffer at a flow rate of 0.35 mL min^{-1}, under monitoring at 280 and 540 nm. The procedures were carried out at 0 - 4 °C.

Prior to spectrophotometric measurements, myoglobin was incubated for 1 h at 5, 20, 30 and 40°C in respected buffers described below. After the preincubation, the samples were

immediately cooled on ice and used for the subsequent experiments.

C. Circular dichroism (CD) spectrometry

Purified myoglobin was intensively dialyzed against 10 mM sodium phosphate buffer (pH 7.0), 0.1 M KCl, 0.1 mM dithiothreitol (DTT), and 0.001% NaN_3 and adjusted to the final concentration of 0.025 mg mL^{-1} for measurement of molar ellipticity at 222 nm ($[\theta]_{222}$) or 1.0 mg mL^{-1} for scanning the spectrum in the range of 200 to 240 nm, respectively. The $[\theta]_{222}$ values were measured in a range of 5 to 95°C with a J-720 spectropolarimeter (JASCO). A quartz cell of 10 mm optical path length was used. The obtained CD data were normalized to a scale of 0 – 1 (corresponding to the ellipticity at 5°C) and approximated. Apparent melting temperature (T_{Mapp}), namely, the temperature with the molar ellipticity being just between folded and unfolded states, was referred to estimate the thermal stability of preincubated myoglobin. Other parameters were calculated as follows.

Other parameters were calculated as follows:

Molar ellipticity $[\theta]$ (deg cm^2 $decimol^{-1}$) = θ / (1000 x c x L), where θ is ellipticity (mdeg), c is molar residual concentration (decimal cm^{-3}), and L is the path length (cm)

Transition temperature T_{Mn} (°C) = ΔH_n / ΔS_n -273.15, where ΔH_n is the enthalpy change (J/mol) and ΔS_n is the entropy change (J/mol・K)

α-Helical content (%) = 100 × ($[\theta]_{222}$ / -36,000)

Myoglobin was not completely unfolded at around the final temperature (95°C). Therefore, the ellipticity at the complete unfolding was estimated by the above calculation. Reliability of deconvolution analysis was verified based on the extinction coefficients obtained through the calculation [18].

D. Absorption spectrometry

Myoglobin (1 mg mL^{-1}) in potassium phosphate buffer (pH 7.0) was scanned in the range of 200 – 700 nm using a V-630 spectrophotometer (JASCO). Measurement was performed at room temperature using a 1 mm light path glass cell.

E. Other analytical methods

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 17.5% gel. Protein concentration was determined by BCA Protein Assay Kit (Thermo), using horse heart myoglobin (Sigma-Aldrich) as a standard.

III. Results

Tuna myoglobin was isolated from the dark muscle of bluefin tuna as reported previously. Single gel filtration chromatography gave myoglobin of high purity (<95%) as demonstrated by SDS-PAGE (data not shown). After checking the purity of myoglobin, it was immediately dialyzed against the buffers used in the subsequent experiments. Myoglobin solution was subsequently subjected to preincubation to cause partial structural changes. In Fig. 1 is shown the CD spectra of the preincubated myoglobin. Based on the decrease in ellipticity, it is evident that the preincubation temperature clearly affected on α-helical content of myoglobin. Preincubation at 20°C resulted in slight increase of helical content. Preincubation at 30 and 40°C, however, reduced the helical content of myoglobin to comparable levels.

To perform thermodynamic analysis of the thermal structural changes of myoglobin in detail, temperature dependence of the molar ellipticity at 222 nm ($[\theta]_{222}$) was examined. As shown in Fig.2A, the ellipticity increased in a temperature depending manner. Dramatic increase in ellipticity occurred at around 80 °C. The differentiated values of ellipticity against temperature increase clearly showed the decay of helical structure at around this temperature (Fig.2B). It is clear that the structure of myoglobin preincubated at 40°C was intensively damaged. On the other hand, only slight differences were recognized in the extent of temperature dependent unfolding, judging from the folding rate (Fig.2C).

By the deconvolution analysis of the thermal structural change profile, thermodynamic parameters were calculated for the thermally treated tuna myoglobin (TABLE I). These values indicated that myoglobin denatured through four steps. The lowest transition temperature (T_{M1}) showed that preincubated myoglobin denatured at much lower temperature compared to other myoglobin. However, the apparent transition temperature (T_{Mapp}) was comparable irrespective of preincubation temperature. The enthalpy differences for each denaturation step (ΔH_n) tended to increase as the preincubation temperature was raised. The apparent free energy for unfolding (ΔG_{app}) of myoglobin showed that myoglobin preincubated at 40°C was most thermostable, because the fragile regions of myoglobin have already been perturbed by the preincubation and only the stable structure remained. On the other hand, the helical content decreased to around 80% by the preincubation at 30 and 40°C.

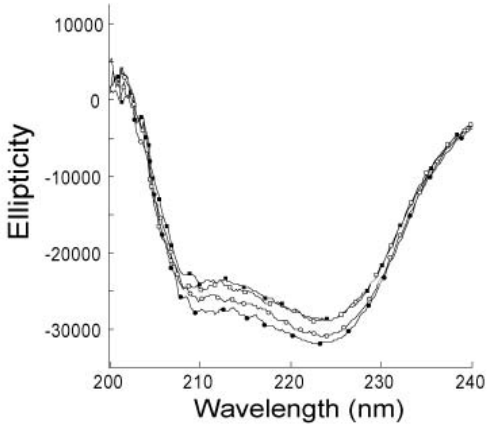

Fig. 1 CD spectra of myoglobins preincubated at respective temperatures. Open circles, filled circles, open squares and filled squares represent the data for myoglobins preincubated at 5, 20, 30 and 40°C, respectively

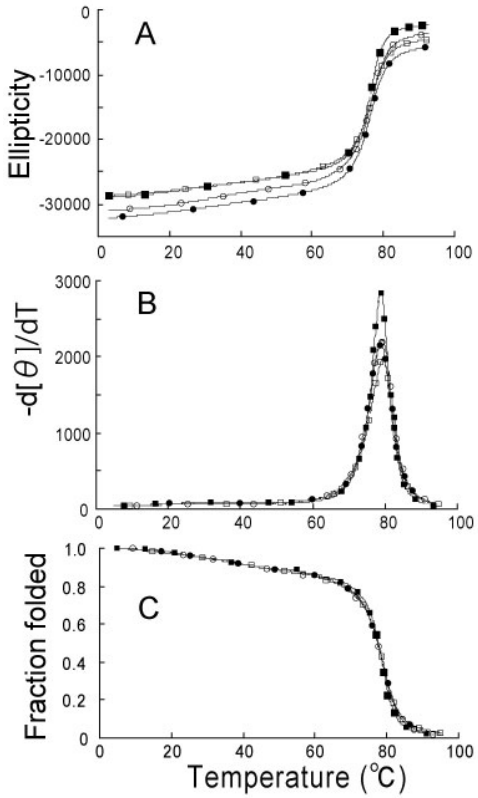

Fig. 2 Molar ellipticity at 222 nm ($[\theta]_{222}$) (A), differential values of ellipticity against temperature (B) and the fraction of folded myoglobin molecule (C) after preincubation. Open circles, filled circles, open squares and filled squares represent the data for myoglobins preincubated at 5, 20, 30 and 40°C, respectively

TABLE I
EFFECT OF PREINCUBATION TEMPERATURE ON THE THERMODYNAMIC PARAMETERS FOR FOLDING OF MYOGLOBIN

	5℃	20℃	30℃	40℃
T_{M1}	37.5	33.4	30.4	24.3
T_{M2}	76.5	75.3	73.5	63.2
T_{M3}	77.8	78.0	78.1	77.3
T_{M4}	80.0	79.2	79.9	79.0
T_{Mapp}	77.5	77.3	77.6	77.7
ΔH_1	-65	-53	-57	-106
ΔH_2	-209	-123	-105	-79
ΔH_3	-611	-392	-396	-330
ΔH_4	-668	-696	-832	-783
ΔH_{total}	-1553	-1264	-1390	-1298
ΔG_{app}	-60.9	-55.0	-57.3	-73.6
α-Helical content	85.6	88.9	80.1	79.3

ΔH is the enthalpy for folding of the helix-coil transitions in kJ/mol. T_M is the observed midpoint of each transition, and T_{Mapp} is the temperature at which the ellipticity, normalized to a scale of 0-1.0, was equal to 0.5. ΔG_{app} is the free energy of folding at 20℃ in kJ/mol. α-Helical content is that at 5 ℃ in %.

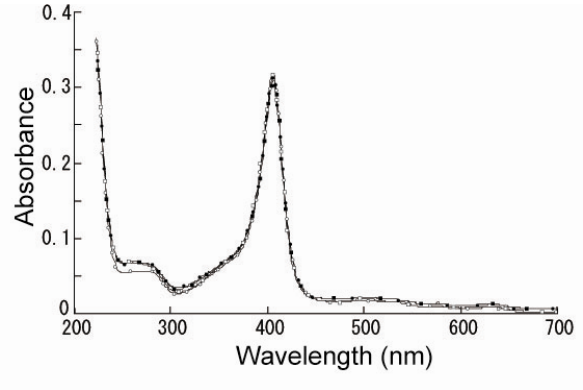

Fig. 3 Ultraviolet and visible spectra of myoglobin after preincubation at respective temperatures. Open circles, filled circles, open squares and filled squares represent the data for myoglobins preincubated at 5, 20, 30 and 40°C, respectively

The effect of preincubation temperature on the ultraviolet/ visible absorption spectra is shown in Fig. 3. The spectra for each sample with different preincubation temperatures were overlaid almost completely, though slight differences were recognized in the spectrum for myoglobin preincubated at 5°C. It is noteworthy that the spectra around the Soret band peak (ca. 409 nm) were quite the same irrespective of preincubation temperature.

IV. Discussion

In the present study, attempts were made to examine the structural instability of tuna myoglobin closely related to its fast autooxidation profile. The purified myoglobin was subjected to partial denaturation by incubation at respective temperatures. Preincubation at 40°C clearly perturbed the structure of myoglobin as observed by CD spectra measurement (Fig. 2B). These values described the structural decay of myoglobin precisely, especially by incubation at 40°C. Body temperature of tunas is considered to be around 30°C [19]. Therefore, it is quite likely that tuna myoglobin did not suffer from thermal structural change by the incubation at this temperature.

Helical regions of myoglobin were also affected by preincubation at higher temperatures, especially at 40°C. Though the helical content was found to be decreased by thermal treatment, the absorption spectra showed that no essential change took place around the Soret band absorption which is closely related to the structural change of heme pocket. It is thus likely that fast autooxidation of tuna myoglobin is caused by only a slight changes of the region(s) other than the heme pocket.

Stability of myoglobin has been investigated by taking various parameters. Fluorescence correlation spectroscopy disclosed the extent of structural change of tuna apomyoglobin [20]. The decrease in Soret band absorption is also a useful parameter for the denaturation of myoglobin [9,10,16]. Soret band absorption is related to the structure around the heme pocket. When the structure is perturbed, the absorbance decreases. In the present study, the absorption spectra of all the samples showed very similar patterns, except that ultraviolet

absorption spectrum at 5℃ was slightly different from each other. The result suggests that the present thermal treatment condition did not cause any observable structural change of the heme pocket. However, based on the previous studies, circular dichroism (CD) and differential scanning calorimetry measurements can offer detailed thermodynamic profiles of proteins to best describe the structural perturbation of myoglobin [5,6,13].

Stability differences of myoglobins are quite species specific. Fish myoglobins are prone to aggregation at high temperatures [5]. The region of 100-114th residues in G helix in human myoglobin was found to aggregate easily [21]. The regions such as residues 9-12, 31-33, 65-70, and 108-114, were also predicted to aggregate by calculation. Some residues were also identified as those responsible for aggregation, namely, Asp5 and Gly6 of A helix, Ala23 of B helix, Gly125, Ala126, and Asp 127 of H helix [22]. In the molten globule state of myoglobin, A, G, and H helices already form a native-like structure and a hydrophobic core [23]. B and E helices also contribute to the core formation in the apomyoglobin. Even the acidic compact state of sperm whale apomyoglobin contains an ordered structure of core formed by A, G and H helices [24].

Regarding the stability difference between fish and mammalian myoglobins, free energy for unfolding yellowfin tuna myoglobin was found to be lower than that of whale myoglobin [25]. Body temperature (or ambient temperature for most fish species) is considered to be especially a critical factor responsible for stability of myoglobin [26,27]. Differences in thermostability were recognized among tuna myoglobins as described above. The amino acid sequence identity between yellowfin tuna and bluefin tuna myoglobins was approximately 98%, suggesting they share almost identical structure and stability, although they show a slightly difference in thermal stability. Among the scombroid fish species examined, skipjack tuna *Katsuwonus pelamis* myoglobin was the most stable, and bullet tuna *Auxis rochei* myoglobin showed the lowest stability [5,6]. Amino acid substitutions were present in A, B, and E helices. Most of the residues controlling the stability of tuna myoglobins have been identified by mutation studies performed by our research group [9,10]. The substitutions in A and E helices were found to greatly affect the thermostability.

Site-directed mutagenesis studies have also shed light on the structural properties of apomyoglobin, molten globule intermediate states, and intramolecular electron transfer, and the effect of heme binding [28,29]. The stability of sperm whale apomyoglobin was increased by replacing Pro88 in helix F by Ala [30]. Mutations of conservative and non-functional Trp14 and Met131 resulted in destabilization, but the substitution of non-conservative Val17 did not result in changes in the cooperativity of the conformational transitions [31]. On the other hand, the stability of sea hare *Aplysia limacina* myoglobin intermediate was decreased by replacing Trp130 by Tyr, which is important for the packing of A, G, and H helices [32]. The proximal His residue contributes to maintain a waterproof environment of heme pocket and prevent the dissociation of hemin from myoglobin [32]. Replacement of Ser92 with Leu or Ala in porcine myoglobin resulted in enhanced dissociation of heme [7]. The mutations of distal His64, Val68, and Arg45 also promoted heme dissociation [34]. F helix is disordered by the removal of heme. All these studies supported the view that quite a limited number of amino acid residues are responsible for the formation of the heme pocket and stabilization of Mb.

Tuna apomyoglobin was characterized by molecular dynamic simulation as well as by spectroscopic analyses [7]. Similar results obtained by the both methods showed that the folding intermediates cannot be explained by a single conformation, but are composed of many conformations which share energetic properties in common. Computer simulation also could reveal that sperm whale apomyoglobin could refold from the thermally denatured state through different folding pathways [35].

V. Conclusion

In the present study, effect of temperature on the structural changes of tuna myoglobin under physiological environment was investigated. It was revealed that tuna myoglobin is stable up to around body temperature, thus the structure of apomyoglobin is not closely related to the fast discoloration rate of fish myoglobin. However, muscle pH is subject to fluctuate through rigor mortis by such reactions as ATP breakdown and lactic acid formation. The effect of pH changes on the myoglobin structure is going to be examined.

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