論 文 の 内 容 の 要 旨

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Studies on the tissue distribution, structure and function profiles of fish myoglobins

（魚類ミオグロビンの組織分布および構造に関する研究）

Myoglobin (Mb), distinguished as the first protein for which the three-dimensional structure was determined, is a small, single subunit intracellular hemoprotein. Mb has been a popular model system to study protein structure, function, dynamics, evolution and expression in relation to its roles in oxygen storage especially during periods of hypoxia or high oxygen demand, oxygen partial pressure buffering and facilitated oxygen diffusion. However, physiological function and localization of Mb, most often considered just an oxygen repository in the oxidative muscle tissues, have been considerably diversified over the last few years and have become the object of renewed interest regarding its potential roles beyond those previously characterized. In the present study, attempts were made to link up the ‘classical’ roles of Mb regarding its structure and function with the ‘contemporary’ concepts of diversification in terms of expression and tissue localization.

Chapter I: cDNA cloning and primary structure of teleost Mb

Primary structure analysis of Mb from different species can help to identify important residues involved in the subtle differences regarding stability and oxygen binding profiles of Mb, and at the same time, can be a reliable guide for assessing evolutionary route of Mb. Therefore,

cDNA sequences of Mb from eleven teleost species representing seven different families were newly determined. cDNAs encoding Mb from these species contained open reading frames of 444 nucleotides encoding 147 amino acids except for rainbow trout *Onchorhynchus mykiss* and walking catfish *Clarias batrachus*, which contained 441 nucleotides encoding 146 amino acid residues. The protein mass and isoelectric point of these Mbs was in the range of 15.5-18.2 kDa and 6.51-9.50, respectively. Apart from heme binding histidines (His60 and His89), a few conserved residues were also recognized, for example, Leu29, Phe30, Phe40, Phe43, Val64, etc. The primary structures of rainbow trout and walking catfish Mbs were exceptional due to residual deletions in the F helix and the presence of additional cysteine residues in the F helix and E-F inter-helical segment, respectively. Despite occasional substitutions all over the molecules, sequence identities among teleost Mbs were high (53-99%), but low when compared with mammalian Mbs (31-41%), and thereby extended the concept that primary structure of Mb is species-specific. Detailed phylogenetic reconstruction of teleost Mbs based on deduced amino acid sequences along with other globin proteins clearly separated teleost Mbs from mammalian Mbs, and subsequently from other globins. Mbs from respective families formed clusters together as revealed by phylogenetic analyses. Phylogenetic distances between teleost and mammalian Mbs reflected the diverse functional properties of respective Mbs, through the adaptation to various living conditions or tissue specific expression during evolution.

Chapter II: Structural and functional characterization of teleost Mbs

To unfold how the primary structure influences the stability and function, Mbs were purified from three carangids, namely, yellowtail *Seriola quinqueradiata*, greater amberjack *Seriola dumerili* and silver trevally *Pseudocaranx dentex*, and their thermostabilities were measured by circular dichroism (CD) spectroscopy. For carangid Mbs, molar ellipticity values at 222 nm ($[\theta]_{222}$) at 10°C were in the range of -22468 to -25084 with calculated α-helical contents of 65.3% to 72%. Despite high sequence homology among carangid Mbs (80%-99%), thermal stability was in the order of silver trevally Mb> yellowtail Mb> greater amberjack Mb. The mammalian reference, horse Mb, displayed higher thermostability ($[\theta]_{222}$= -28029 and α-helical contents= 79.6%) than carangid Mbs and therefore extended the fact that teleost Mbs are unstable than the counterparts of higher vertebrates. On the other hand, autoxidation rate constants of carangid Mbs, plotted as $\ln\{(MbO_2)_t/(MbO_2)_0\}$ versus time were linear, thereby

disclosing that the autoxidation was first order reaction with respect to remaining oxymyoglobin. The observed first order rate constant increased with temperature, reflecting an acceleration of autoxidation under higher temperature. The order of autoxidation rates, irrespective of temperature (either 25°C or 37°C), were silver trevally Mb (0.27 h^{-1} or 0.39 h^{-1}) > yellowtail Mb (0.2 h^{-1} or 0.27 h^{-1}) > greater amberjack Mb (0.14 h^{-1} or 0.18 h^{-1}). These findings suggest that thermal stability and oxidative stability of Mb may not be necessarily correlated.

Chapter III: Structural and functional characterization of rainbow trout recombinant Mb

Considering the uniqueness in the primary structure of rainbow trout Mb, its recombinant expression system was established. By using two different expression vectors (pET-11a [without fusion partner] and pET-32a [with His-tag]) with two different heme biosynthesizers (δ-Aminolevulinic acid and hemin), recombinant Mbs were successfully obtained. Recombinant Mb with the fusion partner displayed somehow non-conformity in the secondary structure pattern, whereas, the recombinant Mb without the fusion partner resembled the secondary structure of recombinant Mbs. Thermostability of rainbow trout recombinant Mb was elucidated by means of CD spectroscopy. Molar ellipticity values of rainbow trout recombinant Mb at 222 nm ($[\theta]_{222}$) was -11044 at 10°C with α-helical content of 36%. A non-cooperative thermal denaturation profile was observed for the recombinant Mb. To explore the presence of free cysteine residue(s) in the primary structure of rainbow trout Mb, the recombinant Mb was reacted with 4, 4´-dithiodipyridine which ultimately resulted in the oxidation of Mb with characteristic increment in the absorption at 324 nm because of the formation of thiopyridine. The result affirmed the presence of free cysteine residue(s). In order to elucidate the reactive oxygen species (ROS) scavenging activity, rainbow trout recombinant Mb was reacted *in vitro* with hydrogen peroxide. Recombinant Mb displayed steady peroxidase reactivity observed by the spectroscopic decay of oxymyoglobin to metmyoglobin, indicating *in vivo* roles of Mb as a ROS scavenger.

Chapter IV: Expression of Mb in muscle and non-muscle tissues of rainbow trout

In recent years, the prevalent understanding of Mb has been gradually, but definitely, changed in terms of distribution pattern and functional diversity. Mb distribution and expression pattern are supposed to be influenced by life style and living environment. In hypoxia tolerant

cyprinids, it has been established that Mb is not restricted only to oxidative muscle tissues. Therefore, investigations were forwarded to explore the expression pattern of Mb transcripts in hypoxia intolerant species, rainbow trout, along with other reference species. Mb transcripts were strongly generated against heart and skeletal muscles, whereas, Mb mRNA levels were comparatively high in gonad and gill among the non-muscle tissues examined. At the protein level, rainbow trout Mb was detected in several non-muscle tissues by Western-blotting. By using RNA *in situ* hybridization and immunofluorescence, rainbow trout Mb was localized in cardiomyocytes and a subset of muscle fibers. On the other hand, expression of rainbow trout Mb was restricted to lamellar epithelial cells, epithelial layers of hepato-biliary duct, neurons and endothelial cells of brain, ooplasm of gonad and so on. Thus, Mb might play different roles with yet unknown physiological significance. Taken together with data reported for cyprinids and Mb localization pattern observed for reference species, it is likely that Mb is ubiquitously expressed in the non-muscle tissues of teleosts with variable amounts depending on the physiology, life style and activity of corresponding species. In rainbow trout brain, neuroglobin, a distant member of globin superfamily, was co-localized at the same cellular sites with Mb indicating that Mb concerting with the other globins might play important physiological role(s).

Finally, the present study renders insight into typical species-specific primary structure, thermostability and autoxidation profile of fish Mb. Establishment of a recombinant expression system and uniqueness of the structure is also evident by the presence of free, reactive cysteine residue and *in vivo* ROS scavenging activity of Mb. As residual substitutions took place all over the Mb molecule, key residues behind variable thermodynamics, oxidative stability and ROS reactivity have been less understood. In this regard, it is interesting to investigate the putative residues by site directed mutagenesis for further information. The present study also highlights the ubiquitous expression pattern of Mb at both mRNA and protein levels in non-muscle tissues of teleost, irrespective of their diverse ecophysiological profiles. Eye catching concurrent occurrence of two different globins at the same cellular site is also evident. Further investigations using gene knockouts would help to understand the cooperativity among globins.