AMINO ACID SEQUENCE OF JAPANESE QUAIL (*COTURNIX JAPONICA*) AND NORTHERN BOBWHITE (*COLINUS VIRGINIANUS*) MYOGLOBIN

by

JOHN ARTHUR GOODSON

(Under the Direction of Anand Mohan)

ABSTRACT

Myoglobin has an important physiological role in vertebrates, and as the primary sarcoplasmic pigment in meat, influences quality perception and consumer acceptability. In this study, the amino acid sequences of Japanese quail and northern bobwhite myoglobin were deduced by cDNA cloning of the coding sequence from mRNA. Japanese quail myoglobin was isolated from quail cardiac muscles, purified using ammonium sulfate precipitation and gel-filtration, and subjected to multiple enzymatic digestions. Mass spectrometry corroborated the deduced protein amino acid sequence at the protein level. Sequence analysis revealed both species' myoglobin structures consist of 153 amino acids, differing at only three positions. When compared with chicken myoglobin, Japanese quail showed 98% sequence identity, and northern bobwhite 97% sequence identity. The myoglobin in both quail species contained eight histidine residues instead of the nine present in chicken and turkey.

INDEX WORDS: Japanese quail, northern bobwhite, cDNA cloning, myoglobin, primary structure, *Coturnix japonica*, *Colinus virginianus*

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JOHN ARTHUR GOODSON

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JOHN ARTHUR GOODSON

Major Professor:	Anand Mohan
Committee:	Rakesh K. Singh
	Robert B. Beckstead

Electronic Version Approved:

Julie Coffield
Interim Dean of the Graduate School
The University of Georgia
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DEDICATION

I would like to dedicate this work to my family and my undergraduate advisors, Dr. Robert Shewfelt and Dr. Marianne Shockley, for their support and understanding.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Presented in this chapter is an overview of myoglobin structure and function and the methodologies and technology involved in the present work. This includes background on the organisms studied, notes on previous investigations into myoglobin primary structure, and effects on meat color.

Myoglobin Protein and Primary Structure

Myoglobin (Mb) is a monomeric protein generally found within the myocyte of vertebrates and serves as the primary pigment in meat. It is typically found in high concentrations within cardiac muscle tissue and oxidative skeletal muscle fibers (Ordway & Garry, 2004). Structurally related to hemoglobin, Mb functions to reversibly bind oxygen, acting as a reservoir of oxygen during extreme conditions such as anaerobic exercise. The Mb of sperm whale was the first protein to have its three-dimensional structure revealed at the atomic level (Kendrew, Dickerson, Strandberg, Hart, Davies, Phillips, et al., 1960). Over half a century later, Mb is perhaps the best-understood protein in terms of molecular structure-function relationships (Gros, Wittenberg, & Jue, 2010). However, despite this, research continues to unveil new discoveries regarding the physiological function of the protein. In 1998, the importance of Mb as a storage facility for oxygen was questioned when it was revealed, in gene-knockout experiments, that

mice lacking the protein showed no obvious deleterious effects during exercise or low-oxygen conditions (Garry, Ordway, Lorenz, Radford, Chin, Grange, et al.).

Aside from the protein's textbook role as an oxygen repository, Mb is also important as a mediator of diffusion of oxygen from the capillaries to the mitochondria, maintaining constant oxygen pressure throughout the cells and allowing for partial saturation of Mb independent of the workload (Wittenberg & Wittenberg, 2003). Studies have also revealed the significance of this role when it was discovered that nitric oxide (NO) irreversibly inhibits cytochrome c oxidase, suppressing the mitochondria and thereby interrupting the respiratory chain (Cleeter, Cooper, Darley-Usmar, Moncada, & Schapira, 1994). Further research established the potential for Mb as a NO scavenger, as oxygenated Mb readily reacts with NO to form nitrate and metmyoglobin (Brunori, 2001). In recent years, Mb's role with NO was expanded by evidence that, under anaerobic conditions, deoxygenated Mb coverts endogenous nitrite to NO (Hendgen-Cotta, Merx, Shiva, Schmitz, Becher, Klare, et al., 2008). Under these hypoxic conditions, NO acts as an activator of signaling pathways responsible for hypoxic vasodilation, and its effect of mitochondrial suppression limits the generation of harmful reactive oxygen species in tissues suffering from ischaemia and from reperfusion damage (Shiva, Huang, Grubina, Sun, Ringwood, MacArthur, et al., 2007). From these newer findings, it can be surmised that Mb plays an essential role in NO homeostasis. There is still much to uncover regarding the complete physiological role of Mb, and studies have found Mb isoforms in a variety of non-muscle tissues such as the brain and liver suggesting versatility in function (Cossins, Williams, Foulkes, Berenbrink, & Kipar, 2009). Furthermore, the functions of related globin proteins such as cytoglobin and

neuroglobin are yet to be established (Gorr, Wichmann, Pilarsky, Theurillat, Fabrizius, Laufs, et al., 2011).

Structurally, Mb consists of a polypeptide chain of 145 to 154 amino acids, depending on the species and whether the starting methionine residue is included, folded into a globular structure with a heme group situated toward the center (Livingston & Brown, 1981). It is the iron-containing porphyrin, heme, which serves as the binding site for oxygen (Ordway & Garry, 2004). The presence of bound oxygen and the oxidation state of the iron atom contribute to the color of the pigment. As described by Young and West (2001), in the reduced, ferrous (+2) state, Mb will take on a purplish-red color in the absence of a bound oxygen molecule and a distinctive bright red color when oxygen is present. In this context, the pigment is known as deoxymyoglobin and oxymyoglobin, respectively. When either forms are oxidized, as is common for meats under long storage periods, metmyoglobin is formed and a brown color is imparted (Young & West, 2001). Discoloration in raw meats, resulting from metmyoglobin formation, is unfavorable to consumers, so methods of stabilizing oxymyoglobin are of great interest to producers (Gray, Gomaa, & Buckley, 1996).

It has been shown that primary structure affects the color stability directly via such mechanisms as structural stability (Ochiai, Ueki, & Watabe, 2009; Regis, Fattori, Santoro, Jamin, & Ramos, 2005), thermostability (Ueki, Chow, & Ochiai, 2005; Ueki & Ochiai, 2006), oxygen affinity (Enoki, Ohga, Ishidate, & Morimoto, 2008), heme retention (Grunwald & Richards, 2006b), autoxidation (Stewart, Blakely, Karpowicz, Kalanxhi, Thatcher, & Martin, 2004), and interactions with aldehydes (Suman, Faustman, Stamer, & Liebler, 2007) and lactate (Tamburrini, Romano, Giardina, & di Prisco, 1999).

These findings are indicative of the ability of variations in Mb primary structure to influence the tertiary structure. Primary structure has also been noted as a possible endogenous factor contributing to pinking defect in poultry due to its major effects on thermostability and interaction with ligands (Holownia, Chinnan, & Reynolds, 2003; Joseph, Suman, Li, Beach, & Claus, 2010). Therefore it is important to characterize the Mb completely in order to understand species-specific variations and Mb chemistry. During the course of this study, the genomes of both Japanese (Kawahara-Miki, Sano, Nunome, Shimmura, Kuwayama, Takahashi, et al., 2013) and northern bobwhite (Halley, Dowd, Decker, Seabury, Bhattarai, Johnson, et al., 2014) quail were published. However, to date, there has been no investigation regarding the complete sequences of Mb in either species.

Primary Structure Investigations in Myoglobin

Mb function is highly conserved across vertebrate species. Despite this, there is enormous variation in the primary structure of globins in general when considered among all reported sequences (Kapp, Moens, Vanfleteren, Trotman, Suzuki, & Vinogradov, 1995). Certain positions within the aligned sequences of Mb proteins have been shown to be less apt to substitution, implying greater importance to the structural stability and/or function of the protein (Laberge & Yonetani, 2007; Lesk & Chothia, 1980; Ptitsyn & Ting, 1999). In recent years, with the advent of more powerful proteomic analysis tools and methods, research into the effects these substitutions have on Mb stability, three-dimensional structure, and its interactions with other molecules has been investigated in various studies, some of which are described as follows.

The structurally important distal (His64) and proximal (His93) histidine residues, responsible for coordination of the heme group, are two of the most conserved residues in Mb (Herold, 2005). However, research conducted by Dene, Goodman, and Romero-Herrera (1980) showed that in elephant Mb distal histidine is substituted by glutamine. The ability of Mb to reversibly bind O_2 is crucial to its primary role. During the process of reversible binding, the ferrous state (+2) of the iron atom must be maintained as the atom cannot bind O_2 in the ferric state (+3), where it is thus biologically inactive (Shibata, Matsumoto, Nishimura, Tai, Matsuoka, Nagao, et al., 2012). However, while oxygenated in the ferrous state, the iron atom is readily oxidized into the ferric state, a process known as "autoxidation" (George & Stratmann, 1954). Subsequent studies on elephant Mb primary structure by Romero-Herrera, Goodman, Dene, Bartnicki, and Mizukami (1981) revealed that the glutamine substitution resulted in a much slower rate of autoxidation in elephant Mb. The predominant physiological effect of substitution was determined to be the maintenance of the reduced state for iron (Bartnicki, Mizukami, & Romero-Herrera, 1983).

Comparative studies into lipid-oxidation induced oxidation have revealed species-specific differences in redox stability that emphasize differences in primary structure as a determining factor. Suman, Faustman, Stamer, and Liebler (2006) reported that beef Mb was more susceptible to such oxidation than pork Mb, both in meat and in vivo (2007). Subsequent investigations involving seven different meat-producing species (beef, sheep, deer, horse, pork, turkey, and chicken) by Yin, Faustman, Tatiyaborworntham, Ramanathan, Maheswarappa, Mancini, et al. (2011) found that oxidation was greater in Mb that contained 12 ± 1 histidine residues (beef, sheep, deer, and horse) than in those

with 9 histidine residues (pork, turkey, and chicken). They concluded that the number of histidine residues within the Mb of a species was directly correlated with a greater redox instability.

Avian and livestock Mb do not contain cysteine residues, and conversely, only one cysteine has been reported in the sequences of tuna, rodent, human, and greater ape Mb (Suman, Joseph, Li, Beach, Fontaine, & Steinke, 2010). Cysteine has shown itself to be a highly oxidizable residue (Carbone, Doorn, Kiebler, & Petersen, 2005). Past investigations on the influence of primary structure on autoxidation by Brown and Mebine (1969) showed that, under equivalent conditions, yellowfin tuna oxymyoglobin oxidized much faster than those from sperm whale or bovine origin. Later research indicated hydrophobicity differences could not fully explain the higher autoxidation of bigeye tuna Mb when compared with sperm whale Mb (Kitahara, Matsuoka, Kobayashi, & Shikama, 1990). Their work suggests that the presence of a highly oxidizable cysteine residue in bigeye tuna Mb could likely responsible for higher propensity for autoxidation.

It is not clear if cysteine in Mb has any generalized role across species. However, investigations by Helbo, Gow, Jamil, Howes, Smulevich, and Fago (2014) may highlight a species-specific role for cysteine in trout and salmon Mb. They proposed that S-nitrosation of Cys107, present in trout and salmon, but absent in tuna, may increase O_2 affinity by relieving protein constraints that limit O_2 entry to the heme pocket of the unmodified Mb. In the same study, they suggested that the physiological effects of this modification appear to be site-specific to Cys107 and occur through selective pressures rather than general thiol modifications.

The thermostability of Mb is an important determinant of denaturation in cooked meat, and in this way impacts color development. Bigeye tuna Mb has been recently characterized by Ueki and Ochiai (2004). The study also compared the thermostability of bigeye tuna Mb to that of other scombridae fish and found that, despite nearly identical primary structures, each species Mb exhibited different thermostability. Subsequent reports by Ueki, Chow, and Ochiai (2005) characterize the Mb of bullet tuna and came to similar conclusions. Further related investigations by Ueki and Ochiai (2006), using amino acid substitution mutants, confirmed suspicions that structural stability, and thus thermostability, was affected by only a few substitutions in the primary structure, specifically those located in the helical segments forming the heme pocket.

Heme retention, a property upon which the redox state and ligand binding ability of Mb is dependent, has been shown to be influenced by the primary structure (Yang & Phillips Jr, 1996). If the heme group cannot be retained in Mb, then the resulting disassociation of the prosthetic group causes discoloration of meat and deterioration of overall meat quality (Richards, Dettmann, & Grunwald, 2005). Loss of the heme prosthetic group is even more important than autoxidation when concerning the determination of the onset of lipid oxidation (Grunwald & Richards, 2006a).

Using recombinant Mb from sperm whale, Grunwald and Richards (2006b) investigated the effects of single amino acid substitutions on heme retention. The recombinant Mb proteins consisted of two groups, one in which histidine 97 was substituted with alanine, and the other substituting valine 68 with threonine. In the latter group, the variant showed higher heme affinity, and was an exceptionally poor promoter of lipid oxidation, than the wild-type. However in the former group, the variant suffered

a decrease in heme affinity, and increased promotion of lipid oxidation, compared with the wild-type. The authors posited that the decrease in heme affinity in the variant was a direct result of the small size of the alanine residue, increasing access to the heme group by water at a higher rate, compared with the relatively larger histidine residue in the wild-type. The ability of the threonine residue to hydrogen bond with water, compared to the nonpolar valine residue, and anchor the heme group was suggested as the cause of the increase in heme affinity in the variant where threonine replaced valine in the wild-type. In summation, these investigations confirmed that even a single substitution in the primary structure of Mb can have large effects on heme retention.

Oxygen affinity, like heme affinity, also influences the redox state of Mb. This is chiefly dependent of the accessibility and size of the heme pocket, traits which have been known to be influenced by primary structure (Brantley, Smerdon, Wilkinson, Singleton, & Olson, 1993). Carver, Brantley, Singleton, Arduini, Quillin, Phillips, et al. (1992) conducted similar studies to those described previously by Grunwald and Richards (2006a). The group constructed mutants from sperm whale Mb substituting leucine 29 with the residues alanine, valine, and phenylalanine. They measured the equilibrium constants for O_2 binding to the Mb variants and found that for the alanine and valine mutants, the constants were relatively the same when compared with the wild-type. However, the phenylalanine mutants showed a 10-fold decrease in O_2 disassociation rate constant, resulting in a markedly reduced rate of autoxidation in Mb. They remarked that the increased bulk of the phenylalanine residue, compared to the wild-type leucine, allowed for increased stabilization of the oxygenated heme group. Carver, et al. (1992) suggested that the selective pressure to conserve leucine 29 in sperm whale Mb was

likely a representation of compromise between the reducing rate of autoxidation and maintaining the ability of Mb to facilitate the rapid release of oxygen during physical activity.

The effect of the Mb amino acid sequence on oxygen affinity was also studied in teleost fish by Marcinek, Bonaventura, Wittenberg, and Block (2001). The regions between helices have been postulated to be significant sites for the modulation of conformational flexibility (Fields & Somero, 1998; Gerstein, Lesk, & Chothia, 1994). The group's investigations in mackerel and bonito Mb suggested that substitutions within these regions, which they both contained, may contribute to increased flexibility that would make the conformational changes in the protein contributing to oxygen escape more likely and possibly result in an increase in the dissociation rate constant and therefore lowering oxygen affinity.

Japanese Quail

Coturnix japonica, commonly known as Japanese quail, is a species of migratory bird originating from East Asia of the order Galliformes (Wakasugi, 1984). Japanese Quail are grouped within the family Phasianidae, the largest branch of the order Galliformes, along with chicken, turkey, pheasant, partridge, grouse, and other Old world quail (Cox, Kimball, & Braun, 2007). Originally domesticated as pets and song birds, the earliest historical records of domesticated Japanese quail have been found from twelfth century Japan (Howes, 1964). They were likely first domesticated there during the eleventh century there or imported already in domesticated form from China (Howes, 1964). Japanese quail are an attractive alternative farm animal with respect to its small body size, quick growth, ease of management, high egg production and feed-to-egg

conversion ratio (Cheng, Bennett, & Mills, 2010; Shanaway, 1994). In recent decades, commercial quail farming has been developed across many countries (Minvielle, 2004). Within the consumer market, quail is generally regarded as a gourmet or ethnic food, and currently makes up a miniscule percentage of poultry sales in comparison to broilers (da Cunha, 2009). However, there is growing consumer interest in the quail as an alternative to chicken and turkey in western markets, and research into quail meat composition and quality shows nutritional advantages when compared to other poultry birds (Genchev, Mihaylova, Ribarski, Pavlov, & Kabakchiev, 2008). In commercial production, Japanese quail mature at about 5 weeks of age at an average weight of about 215 g (Cheng, Bennett, & Mills, 2010). These qualities have also lent Japanese quail to be a popular laboratory animal, and it has been used as a model organism in many research studies including the fields of genetics, endocrinology, nutrition, physiology, toxicology, embryology, and gerontology (Cain & Lien, 1985; Flachowsky, Halle, & Aulrich, 2005; Li, Zhu, Wang, Zhu, Chang, & Kritchevsky, 1998; Muller, Zahn, Schroder, & Arendes, 1979; Shih, Pullman, & Kao, 1983; Yamamura, Hirunagi, Ebihara, & Yoshimura, 2004; Zile, 1998).

Northern Bobwhite Quail

Northern bobwhite quail (*Colinus virginianus*), another species of domesticated quail native to North America, was once thought to be closely related to Japanese Quail, and was positioned under Phasianidae as a subfamily group (Johnsgard, 2008). However, newer evidence has shown that New World quail, such as the northern bobwhite, likely owe their similar appearance more to convergent evolution rather than being particularly closely related, and thusly they have been placed in their own separate family,

Odontophoridae (Cox, Kimball, & Braun, 2007). Unlike Japanese quail, they are non-migratory and native to the western hemisphere, including most of North America including southern Canada, eastern and central United States, Mexico, and the Caribbean (Dimmick, 1992). Commercially, within the United States, northern bobwhite are considered game birds, and therefore are mostly bred for hunting purposes (Judd, 1905). In research, northern bobwhites have played a large role in captive wildlife studies concerning the physiological and behavioral effects of pesticides (Vyas, 1999). They also were the subject of the first modern systematic study of a wild animal's life history in relation to habitat and environmental factors that influence its abundance (Dimmick, 1992).

cDNA Cloning

Since its first reporting in 1972, cDNA cloning has become a powerful instrument in the isolation, characterization, and analysis of genes (Verma, Temple, Fan, & Baltimore, 1972). cDNA cloning is a technique that is often employed in order to obtain a sequence of DNA that directs the production of a specific protein. There are numerous techniques that exist in performing cDNA cloning (Coleclough, 1987; Efstratiadis, Kafatos, & Maniatis, 1977; Gubler & Hoffman, 1983; Heidecker & Messing, 1987; Okayama, Kawaichi, Brownstein, Lee, Yokota, & Arai, 1987). The following describes that which was utilized in the present study. In the first step of cDNA cloning, a population of mRNA, including the mRNA coding for the protein of interest, is isolated and converted into cDNA by reverse transcription polymerase chain reaction (RT-PCR) (Bonham, Zafarullah, & Gedamu, 1987; Gubler & Hoffman, 1983). Mb is commonly

found in cardiac and skeletal muscle tissues, and thusly total RNA was isolated from quail breast tissue in the present study (Ordway & Garry, 2004).

RT-PCR is performed by the combined actions of reverse transcriptase and DNA polymerase. The reverse transcriptase synthesizes the first single-stranded cDNA from the mRNA (Verma, 1977). Subsequently, the remaining second strand is created by DNA polymerase to complete the double-stranded cDNA product (Efstratiadis, Maniatis, Kafatos, Jeffrey, & Vournakis, 1975). This occurs across the entire isolated population of mRNA, creating a pool of cDNA clones, or what is known as a cDNA library (Sim, Kafatos, Jones, Koehler, Efstratiadis, & Maniatis, 1979). The resulting cDNA library should contain the cDNA corresponding to the mRNA that encodes for the protein of interest (McClean, 1997). Using polymerase chain reaction (PCR) sequence-specific primers, a piece of known sequence cDNA can be amplified and isolated (Belyavsky, Vinogradova, & Rajewsky, 1989). As highlighted by Huang, Chen, and Jong (2003), it was often difficult, previously, to PCR the entirety of an unknown sequence of a cDNA copy of mRNA. They continued that, in order to overcome such difficulties, certain techniques such as anchored-PCR and inverse-PCR have been developed to overcome this issue. In the present study, however, the highly homologous cDNA for chicken (*Gallus gallus*) Mb was previously reported (Boardman, Sanz-Ezquerro, Overton, Burt, Bosch, Fong, et al., 2002). This allowed for the design and screening of primers with sufficient specificity to adhere to the intended sequence.

As described in Alberts, Johnson, Lewis, Raff, Roberts, and Walter (2002), once the cDNA has been amplified and isolated, it is possible to incorporate it into a plasmid vector, which can then be used to transform bacteria, which can make many copies of the

recombinant plasmid. Once the bacteria are lysed and the plasmids purified, they can then be sequenced to provide the cDNA nucleotide sequence, which corresponds to the mRNA of the protein of interest, free of the introns present in genomic DNA (Alberts, Johnson, Lewis, Raff, Roberts, & Walter, 2002). From this point it is simple to deduce the amino acid sequence of the protein by translating the sequence from the open reading frame of the coding sequence. It is important to note, however, that post-translational modifications cannot be predicted from deduced amino acid sequence (Rosenberg, 1996). Such modifications may alter the mature protein sequence in the present expression systems, although no such case of this has been found in vertebrates (Ueki & Ochiai, 2005).

Matrix Assisted Laser Desorption/Ionization

The present study makes use of a device which has become nearly ubiquitous in protein analysis, the matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometer. MALDI was first introduced in the late 80s by Karas and Hillenkamp (1988) and as a powerful analytical tool. MALDI makes use of a "soft" ionization technique, similar to electro-spray ionization, which allows for the analysis of biomolecules and large organic molecules that tend to be sensitive and unstable when subjected to other methods of ionization (Kaufmann, 1995). The MALDI technique, as described by Stults (1995), is generally performed as follows: In brief, an ultraviolet laser is pulsed at the sample, which is mixed into a solution of matrix material composed of crystalized molecules and organic solvents. The energy of the laser allows for the evaporation of the solvents and the recrystallization of the matrix, which has now become

co-crystalized, with the sample analytes embedded within the crystals. Continued pulsing of the now dried spot causes desorption and ionization of the analytes.

Numerous advantages of MALDI include high sensitivity, ease of use, speed, and resolution. When coupled to a TOF mass spectrometer, the ions, previously vaporized by MALDI, are drawn through the device by an electrical field that accelerates them toward a detector plate (Cotter, 1993). The ions become separated by their velocity, which is dependent on their mass-to-charge ratio, with lighter ions travelling faster (Cotter, 1993). Once the particles reach the detector, this ratio can then be calculated for the ions.

Peptide Mass Fingerprinting

One of the ways that MALDI-TOF mass spectra can be utilized is in an analytical technique known as peptide mass fingerprinting (PMF). Used in proteomics as a method for protein identification, PMF makes use of a database of known protein sequences (Henzel, Watanabe, & Stults, 2003). A protein of interest is first isolated and cleaved using any number of techniques, but usually by digestion with a proteolytic enzyme such as trypsin (Kellermann, 1999). After analysis of the resulting peptides by MALDI-TOF, the spectra can then be compared using the database that theoretically cleaves the available, known protein sequences according to the cleavage method used. The resulting peptides give a specific pattern under MALDI-TOF MS for the protein that is called a "peptide map" (Ingram, 1958). Although, the actual spectra may show incomplete coverage of the protein of interest, enough of the peptides may correspond to a specific protein to provide a reasonable match among a list of probable candidates (Thiede, Höhenwarter, Krah, Mattow, Schmid, Schmidt, et al., 2005). Mascot is one such software search engine that can interpret mass spectrometry data and find potential

matches, and is currently widely used by research facilities worldwide (Weatherly, Atwood, Minning, Cavola, Tarleton, & Orlando, 2005).

There are, however, several limitations involving the use of PMF. Most PFM algorithms require that a protein be completely isolated and therefore assume that the peptides present are all derived from a singular protein (Baldwin, 2004). This is often achieved by purifying a protein sample beforehand using methods such as 1 or 2D polyacrylamide gel electrophoresis (Baldwin, 2004). Contaminated samples, often an issue in PFM, frequently occur due to exposure to keratins and other proteins associated with handling of samples. Unintended proteins in the sample can complicate and possibly compromise analysis results. Another caveat of PMF is that the protein sequence must be present in the database of interest for correct identification (Henzel, Watanabe, & Stults, 2003). In the case of the present study, the Mb sequences of Japanese quail and northern bobwhite have not yet been characterized. In such a case, use of PMF will likely yield a highly homologous protein such as chicken Mb as a result. Depending on the organisms studied, the possibility and reliability of cross-species identification through PMF-matching varies greatly (Mezhoud, Praseuth, Marie, Puiseux-Dao, & Edery, 2009). In cross-species analyses, peptides that are not matched in PMF are likely to include substitutions or post-translational modifications, making it unreliable when comparing species with low homology and sequence conservation (Wilkins & Williams, 1997). These peptides can be sequenced through further techniques such as Edman degradation or MALDI-TOF/MS which further couples the MALDI-TOF instrument to a tandem mass spectrometer (Liska & Shevchenko, 2003).

Although PMF cannot typically be used to characterize the structural sequence of novel proteins, because of the growing availability of sequences in databases, such as the dozens of reported avian Mb sequences, like that of chicken, a large amount of the peptides from novel proteins can be successfully identified. As described by Liska and Shevchenko (2003), it can be assumed that in such novel proteins where there are likely to be few amino acid substitutions with a homolog of a closely related species, that the majority of sequence can be identified through PMF. In this way, PMF may be viable as a method of providing support for a proposed sequence. This could be further enhanced by increasing sequence coverage using overlapping enzymatic peptide sequences, as was pursued in the present study.

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CHAPTER 2

AMINO ACID SEQUENCE OF JAPANESE QUAIL (*COTURNIX JAPONICA*) AND NORTHERN BOBWHITE (*COLINUS VIRGINIANUS*) MYOGLOBIN[1]

[1] John Goodson, Robert B. Beckstead, Jason Payne, Rakesh K. Singh, and Anand Mohan. Submitted to *Food Chemistry*.

Abstract

Myoglobin has an important physiological role in vertebrates, and as the primary sarcoplasmic pigment in meat, influences quality perception and consumer acceptability. In the present study, the amino acid sequences of Japanese quail and northern bobwhite myoglobin were deduced by cDNA cloning of the coding sequence from mRNA. Japanese quail myoglobin was isolated from quail cardiac muscles, purified using ammonium sulfate precipitation and gel-filtration, and subjected to multiple enzymatic digestions. Mass spectrometry was performed to corroborate the deduced protein amino acid sequence at the protein level. Sequence analysis revealed both species' myoglobin structures consist of 153 amino acids, differing at only three positions. Comparisons with chicken myoglobin revealed high similarity, with Japanese quail and northern bobwhite having 98 and 97% sequence identity, respectively. Both quail species'myoglobin contained one less histidine residue than the nine present in chicken.

Introduction

Myoglobin (Mb) is a sarcoplasmic oxygen-binding heme protein primarily responsible for poultry meat color and visual appearance. Depending on the species, Mb consists of a polypeptide chain of 145 to 154 amino acids folded into a globular structure with a heme group situated toward the center (Livingston & Brown, 1981). Pigment is influenced by the presence of bound oxygen and the degree of oxidation of the heme group, which can assume various distinct redox states (Young & West, 2001). Differences in the primary structure of Mb may influence the mechanistic properties of the protein and the overall stability of the various redox states (Suman, Joseph, Li, Steinke, & Fontaine, 2009). Additionally, changes in the amino acid sequence of Mb can influence molecular interactions with ligands and small biomolecules like lactate, which may alter the oxygen binding affinity of the protein (Suman & Joseph, 2013).

Japanese quail (*Coturnix japonica*) is a species of Old World (Phasianidae) migratory quail originating in East Asia and originally domesticated in Japan (Wakasugi, 1984). Northern bobwhite quail (*Colinus virginianus*) is another species of quail similar in appearance to Japanese quail; they are, however, non-migratory and native to most of North America, including southern Canada, eastern and central United States, Mexico, and the Caribbean (Dimmick, 1992). Despite their similar appearance, bobwhite quail are not particularly closely related to Old World quail, and as such are grouped in a separate family, commonly known as New World quail (Odontophoridae) (Cox, Kimball, & Braun, 2007). Commercially, within the United States, northern bobwhite quail are considered game birds and are, therefore, mostly bred for hunting (Judd, 1905). In the consumer market, quail is generally regarded as a gourmet or ethnic food, but consumer interest is growing in quail meat as an alternative to chicken and turkey.

Genchev, Mihaylova, Ribarski, Pavlov, and Kabakchiev (2008) stated that quail meat composition and quality has nutritional advantages over other poultry birds.

Chicken and turkey belong to the same order as Japanese and bobwhite quail (Galliformes), and recent research has shown the primary structure of turkey Mb is identical to chicken (Joseph, Suman, Li, Claus, Fontaine, & Steinke, 2011). The Mb of many domesticated meat and poultry animals have been characterized and studied (*www.expasy.org; www.ncbi.nlm.nih.gov*). To date, however, the amino acid sequences of Japanese and bobwhite quail Mb have not been determined. Therefore, the objective of this study is to characterize the amino acid sequence of Japanese and northern bobwhite quail Mb.

Materials and Methods

RNA Isolation and cDNA Synthesis

The study was conducted using adult quail weighing less than 250 g. All animals were handled and managed in compliance with the internal guidelines of the European Union Directive (2010/63/EU) on the protection of animals used for scientific purposes. Housing and procedures performed on quail were approved by the Institutional Animal Care and Use Committee at The University of Georgia. Live Japanese quail (Quail International, Greensboro, GA) and northern bobwhite quail (University of Georgia, Athens, GA) specimens were sacrificed by cervical dislocation, and 50-100 g of breast muscle tissue samples were collected. Total RNA was extracted using TRIzol (Life Technologies, Carlsbad, CA) in accordance with the manufacturer's protocol. A 20 μL aliquot of total RNA was used with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) to synthesize cDNA. The reaction was carried out at 37°C for 2 hr and terminated by 5 min of heat inactivation at 85°C.

cDNA Cloning of Japanese Quail Mb

Table 2.1 shows nucleotide sequences of primers used for polymerase chain reaction (PCR). Primers JQF and JQR were designed using the nucleotide sequence of the conserved internal regions of chicken Mb to amplify the coding regions of Japanese quail Mb. PCR was run with the designed primers for 35 cycles using a Veriti (Applied Biosystems, Foster City, CA) 96-well thermal cycler. Initial denaturation occurred for 30 s at 98°C. Each thermal cycle consisted of denaturation for 5 s at 98°C, annealing for 5 s at 55°C, extension for 10 s at 72°C, and a final extension for 1 min at 72°C. PCR amplification products were validated by analysis of amplicon molecular weight on 2% agarose gel electrophoresis.

PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The purified DNA was then ligated using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific Inc., Waltham, MA) in accordance with the manufacturer's instructions.

The ligation mixture was used directly for transformation of GC10 competent cells (Sigma-Aldrich, St. Louis, MO). A 1 μL amount of the ligation mixture was added to a 50 μL volume of competent cells on ice for 15 min. The mixture was heated at 37°C for 45 s and placed on ice for 2 min to heat shock the cells. Cells were plated to lysogeny broth (LB) agar (ampicillin 100 µg/mL) plates and incubated at 37°C for 16 hr. Colonies were picked from the plates and analyzed using colony PCR/restriction analysis. Validated colonies were transferred to LB tubes and of 1 μL/mL ampicillin was added. The tubes were incubated for 16 hr, and plasmids were isolated from the liquid cultures using the QIAGEN Plasmid Mini Kit (Qiagen, Valencia, CA) consistent with the manufacturer's protocol. The purified DNA from the picked colonies was sequenced at the Georgia Genomics Facility (Athens, GA), using the BigDye

terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on a 96-capillary 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA).

cDNA Cloning of Northern Bobwhite Mb

Primers (JQF and JQR) previously created for Japanese quail unfortunately failed to amplify the cDNA of northern bobwhite quail. Therefore two sets of primers (BWX1 and BWX3) were designed using the nucleotide sequence for chicken Mb to amplify the areas of the sequence directly surrounding the 5' and 3' ends of the northern bobwhite Mb coding region, which are highly conserved. PCR was run as previously detailed, except the annealing temperature was decreased to 50°C. Amplicons were then purified, ligated, cloned, and sequenced as previously described. The sequences of the amplicons were used to design sequence-specific primers (BWWF and BWWR) to PCR amplify the entire coding region of northern bobwhite Mb. PCR settings were identical to what was previously stated, although annealing temperature was raised to 57°C and extension time prolonged to 15 s. PCR purification, ligation, cloning, and sequencing were carried out as previously described.

Sequence Alignment and Analysis

DNA sequences from Japanese and northern bobwhite quail were aligned using ApE software (*http://biologylabs.utah.edu/jorgensen/wayned/ape/*) to ensure fidelity of the sequence, because reverse transcriptase lacks proofreading capabilities and is therefore error-prone (Varela-Echavarría, Garvey, Preston, & Dougherty, 1992). The translated and deduced protein sequences were aligned using Clustal Omega (*http://www.ebi.ac.uk/Tools/msa/clustalo/*). Molecular masses and isoelectric points of Mb proteins were calculated using Compute pI/Mw (*http://web.expasy.org/compute_pi/*).

Isolation of Japanese Quail Mb

Mb was purified according to the methods used by Cameron Faustman and Phillips (2001) with minor modifications. Briefly, fresh Japanese quail hearts were collected from Quail International, Inc (Greensboro, GA) and frozen at -40°C until further analysis was performed. The frozen hearts were thawed at 4°C and trimmed of visible fat and connective tissue. The cardiac muscle was homogenized in homogenization buffer (20 mM ammonium bicarbonate, pH 9.0, 4°C) at a 1:1 (w/w) ratio and centrifuged at 5000 × g for 10 min. The supernatant was brought to 50% ammonium sulfate saturation and further centrifuged for 20 min at 18,000 × *g*. The resulting supernatant was brought to complete ammonium sulfate saturation and further centrifuged for 1 hr at 20,000 × g. The precipitate was resuspended in the homogenization buffer and dialyzed for 24 hr against the same buffer at 4°C. The dialyzed sample was loaded into a Sephacryl S-200 HR gel-filtration column (2.5 x 100 cm, GE Healthcare, Piscataway, NJ) equilibrated with the homogenization buffer using a peristaltic pump (NE-9000, New Era Pump Systems, Inc., Farmingdale, NY) at a flow rate of 60 mL/h. Mb containing fractions were collected, pooled, and concentrated by saturation with ammonium sulfate, centrifugation, and dialysis.

To further isolate and determine the quality of the prepared protein, the resulting samples were subjected to SDS-PAGE. Samples were prepared with 4× Laemelli sample buffer (277.8 mM Tris-HCl, pH 6.8, 4.4% LDS, 44.4% (w/v) glycerol, 0.02% bromophenol blue) at a 3:1 ratio (v/v) and heated for 5 min at 95°C. Multiple 10 μL aliquots and a commercially available protein standard ladder were loaded onto an 18% polyacrylamide Tris-HCl precast gel and run in a Criterion cell unit (Bio-Rad, Hercules, CA) held at 200 v for 1 hr. Gels were stained with Biosafe Coomassie (Bio-Rad, Hercules, CA), and bands were sliced for in-gel digestion.

Japanese Quail Mb Mass Spectrometric Analysis

Gel isolated Japanese quail Mb was subjected to enzymatic digests using trypsin, endoproteinase Asp-N, or endoproteinase Glu-C. Enzymes were selected based on probable sequence coverage, peptide length, site-specific cleavage, and degree of overlap between the peptides. Individual digests were analyzed using Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF). Tryptic digestions and MALDI-TOF analysis were performed at the University of Georgia Proteomic and Mass Spectrometry core facility in Athens, GA, USA.

Tryptic Digestion

Trypsin digestion and peptide extraction were performed using the following procedure. For 20 min, gels plugs were destained in 80 μL of 50 mM ammonium bicarbonate containing 50% methanol and repeated until the stain was not visible. After removing the destaining solution, 80 μL of 75% acetonitrile (ACN) was added; after 15 min, the ACN was removed. Gel pieces were incubated at 38°C for 20 min. The plugs were treated with 80 μL of 8 mg dithiothreitol (Fisher Biotech, Fairlawn, NJ) in 1 ml of 20 mM ammonium bicarbonate for 1 hr at 38°C. The supernatant was removed and 80 μL of an iodoacetamide solution [18.3 mg of iodoacetamide (Bio-Rad, Hercules, CA) in 1 ml of 20 mM ammonium bicarbonate] was added. This was allowed to react in the dark for 30 min at room temperature. After the supernatant was removed, gel plugs were washed with 80 μL 50 mM ammonium bicarbonate containing 50% methanol for 20 min and then with 80 μL of 75% ACN for 20 min. Washed gel pieces were isolated and dried for 20 min at 38°C. A 100 μg of mass spectrometry grade Trypsin Gold from Promega was solubilized in 100 μL of trypsin resuspension buffer (Promega, Madison, WI) and 5 μL of the resulting trypsin solution aliquot was put into tubes. Before use, 500 μL of 20 mM ammonium bicarbonate was added to each tube, and 30 μL of this solution was transferred to

tubes containing the gel pieces. The tubes were placed in a 38°C incubator for 22 hr. The supernatant was collected after digestion, and the peptides were extracted using, 50 μL of 50:50 ACN and 0.1% trifluoroacetic acid. The reaction mixture was incubated for 20 min, peptide containing solutions pooled, and the procedure was repeated.

Pooled supernatants were dried using a Savant SVC-100H speed vacuum centrifuge system (Savant Instruments Inc., Farmingdale, NY). Samples were submitted for peptide mass fingerprinting and to determine molecular masses of separated peptides using a Bruker Daltonics Autoflex (Breme, Germany) in reflectron mode. A 1-2 μL of 10% ACN with 0.1% trifluoroacetic acid was used to reconstitute the samples, and a spot was placed on the plate. A matrix (0.75 μL) was added, and the spot was allowed to dry. The matrix consisted of 5 mg of alpha-cyano-4-hydroxycinnamic acid in 4:3:1 ACN:0.1% trifluoroacetic acid:48 mM ammonium phosphate in 0.1% trifluoroacetic acid. Data was internally calibrated using autodigestion peaks where they were available, and, if they were not available, statistical calibration was applied. The molecular masses of the peptides from Japanese quail Mb were compared using Mascot software (Matrix Science, London, UK) analysis across other avian species in the NCBInr database. Presence of expected peptides was verified by generating a list of molecular masses using Peptide Mass (http://web.expasy.org/peptide_mass/) and comparing them against those reported by MALDI-TOF analysis. Carbamidomethyl (C) was selected as a fix modification and Oxidation (M) as a variable modification. The peptide tolerance was set to 0.2 Da, and the number of missed cleavages ranged from 0-1.

Endoproteinases Glu-C and Asp-N Digestions

Digestions using endoproteinases Glu-C or Asp-N occurred as follows. Sliced gel bands were prepared for digestion as described in Rosenfeld, Capdevielle, Guillemot, and Ferrara

(1992) with the following modifications. The initial wash of the gel pieces was repeated twice at room temperature. Destained gel pieces were dehydrated by adding 100 μL ACN for 5 min and allowing the gel pieces to air dry for 20 min. Gel pieces were then subjected to enzymatic digestion using either endoproteinase Asp-N or Glu-C at a final protein ratio of 1:50 (w/w).

Digestions were carried out for 18 hr at 37°C in digestion buffer (25 mM ammonium bicarbonate, pH 7.8). Digestion supernatants were collected following digestion, and peptides extractions were performed as follows. Thirty microliters of 25 mM ammonium bicarbonate was added to the gel pieces for 20 min and supernatants were collected. A 1:1 solution of 25 mM ammonium bicarbonate to ACN was added for 20 min, removed, and saved to previous collections. A 30 μL of 5% formic acid solution was added, and after 20 min an equal amount of ACN was also added; after another 20 min, the supernatant was isolated. The prior step was repeated, and the combined collection of peptide-containing supernatants was dried, reconstituted, and analyzed as previously described. This process was repeated for Glu-C digestion, although the concentration of ammonium bicarbonate in the digestion buffer was increased to 100 mM to increase the rate of proteolytic cleavage.

Results and Discussion

Other than chicken and turkey, which share an identical amino acid sequence, currently no other members of the species of the Galliformes order have Mb sequences reported (www.ncbi.nlm.nih.gov). In our study, total RNA of both Japanese and northern bobwhite quail was isolated, and the *c*DNA was synthesized. *c*DNA cloning of the Mb coding regions was performed, and multiple clones of each organism were sequenced. The amino acid sequences for Japanese and northern bobwhite quail are in Figure 2.1. Mb of Japanese quail was isolated via SDS-PAGE and subjected to multiple enzymatic digests. MALDI-TOF analysis of the Japanese

quail Mb digests was compared to the probable fragmentation of the deduced amino acid sequence from each digest (Figure 2.2). Sequence coverage of the detected peptides from MALDI-TOF is in Figure 2.3. Total coverage of the expected sequence was achieved, and the amino acid sequence at the protein level was assembled by overlapping the peptide sequences and comparing homology with previously reported avian myoglobin from other species. The deduced amino acid sequence from Japanese quail *c*DNA cloning was aligned and was, as expected, 100% identical. Northern bobwhite quail Mb could not be subjected to similar analysis because specimen availability was limited.

Japanese quail and northern bobwhite Mb comprise 153 amino acid residues, consistent with other avian and mammalian Mb previously reported (*www.expasy.org; www.ncbi.nlm.nih.gov*). As calculated from the deduced sequences, the molecular mass of Japanese quail Mb was 17,340, and northern bobwhite quail Mb was 17,294 Da. These values are comparable to other avian species and notably close to chicken and turkey (17,290 Da) (Joseph, Suman, Li, Claus, Fontaine, & Steinke, 2011).

Mb thermostability is highest when the pH is near the isoelectric point (Grajales-Lagunes & de Anda-Salazar, 2010). When pH decreases, Mb is more susceptible to denaturation. The deduced sequences in our study show the calculated isoelectric points of Japanese quail and northern bobwhite Mb were identical at 8.71. For comparison, the calculated isoelectric points for chicken was 8.10, for duck 8.71, ostrich 8.71, bovine 6.97, horse 7.36, pig 6.83, and yellowfin tuna 9.00. Interestingly, Japanese and northern bobwhite quail share identical calculated isoelectric points with duck and ostrich, and all four were higher than chicken, which was similar to that of bovine and pig.

The sequence of Japanese quail Mb differed from northern bobwhite Mb at only three locations (12, 122, and 145). In the Japanese quail, Thr12 was substituted with Ser12. At positions 122 and 145, the serine residue in Japanese quail has been replaced by alanine in Northern bobwhite. When comparing Japanese quail Mb to chicken, again only three substitutions (34, 44, and 122) occurred. Northern bobwhite quail Mb contained an additional substitution, and therefore differed from chicken Mb at four locations (12, 34, 44, and 145). His34 of chicken Mb was substituted with Arg34 in Japanese quail. In bobwhite quail the same substitution occurred. Both Japanese quail Mb and northern bobwhite Mb contained glutamic acid at position 44, which is aspartic acid in chicken Mb. As with Japanese quail, the Thr12 in chicken has been substituted with Ser12, and the serine at position 145 in chicken was replaced in bobwhite with an alanine. Lastly, an alanine residue at location 122 in chicken, as for bobwhite quail, was substituted with serine in Japanese quail.

The amino acid sequence identity of Japanese and northern bobwhite quail Mb was compared to other Mb (Table 2). With only three substitutions, the sequence of Japanese quail Mb showed 98.0% homology with both chicken and northern bobwhite Mb. The additional substitution between chicken and bobwhite resulted in a slightly lower sequence identity of 97.4%. The apparent high homology of the Japanese quail and northern bobwhite Mb to chicken was predicted as they share the same taxonomic rank (Order Galliformes). Among the selected avian species, sequence identity was high, as expected, with penguins having the lowest sequence identity with Japanese and bobwhite quail. Compared with the selected sequences of mammals (pig, horse, bovine, sheep, and goat), Japanese and bobwhite quail Mb showed sequence identity ranging from 76.5 for pig to 71.9% for goat.

The structurally important distal (His64) and proximal (His93) histidine residues, responsible for coordination of the heme group, were conserved in both Japanese and bobwhite quail. In chicken Mb, Naveena, Faustman, Tatiyaborworntham, Yin, Ramanathan, and Mancini (2010) revealed that the distal and proximal histidines were covalently adducted by 4-hydroxy-2-nonenal (HNE), a model prooxidant aldehyde and a secondary product of lipid oxidation that can compromise myoglobin redox stability. Several studies have shown histidine is a target of adduction by HNE (Alderton, Faustman, Liebler, & Hill, 2003; C. Faustman, Liebler, McClure, & Sun, 1999; Suman, Faustman, Stamer, & Liebler, 2007; Uchida & Stadtman, 1992). Further studies reacting several well-characterized Mb to HNE indicate that the number of histidine residues may influence susceptibility to the redox destabilizing effect of HNE in species-specific manner (Yin, Faustman, Tatiyaborworntham, Ramanathan, Maheswarappa, Mancini, et al., 2011). Notably, Japanese and bobwhite quail contain 8 histidines (positions 24, 26, 36, 64, 82, 93, 97, and 120) compared to 9 in chicken at the same positions. However chicken contains an additional histidine residue at position 34. The substitution of His34 in chicken with Arg34 in quail may indicate Japanese and northern bobwhite quail Mb is less susceptible to lipid oxidation-induced oxidation than chicken Mb. Although His34 is apparently not a target of adduction in chicken or turkey, HNE adduction has been recently studied in emu, where adduction of His34 was confirmed (Nair, Suman, Li, Joseph, & Beach, 2014).

Note that Japanese and northern bobwhite quail Mb are both devoid of cysteine residues, which are also absent in other avian species and most mammal Mb. Research indicates that the increased autoxidation rate of tuna Mb, compared to beef and sperm whale Mb, was due to the presence of a highly oxidizable cysteine residue (Carbone, Doorn, Kiebler, & Petersen, 2005). Although a general role for cysteine in Mb is not well understood, Helbo, Gow, Jamil, Howes,

Smulevich, and Fago (2014) proposed that S-nitrosation of Cys107, present in trout and salmon, but absent in tuna, may increase O_2 affinity by relieving protein constraints that limit O_2 entry into the heme pocket of the unmodified Mb. In the same study, they suggested that the physiological effects of this modification appear to be site-specific to Cys107 and occur through selective pressures rather than through general thiol modifications.

Recently, Ueki and Ochiai (2004) characterized the Mb of bigeye tuna using methods similar to our study. They also compared the thermostability of bigeye tuna Mb to that of other scombridae fish and found that despite nearly identical primary structures in yellowfin (95.2%) and albacore tuna (98.6%), and completely identical primary structures with bluefin tuna, each exhibited different Mb thermostability. In further studies, they concluded that structural stability was affected by only a few substitutions in the primary structure (Ueki & Ochiai, 2006). Thus, despite high homology between the Galliformes, Japanese and bobwhite quail Mb may have different structural stability.

The primary aim of our study was to characterize the primary structures of Japanese and northern bobwhite quail. Japanese and northern bobwhite quail Mb show high sequence identity with each other, and similarly high sequence identity with chicken Mb. Primary structure dictates tertiary structure, so further investigations should examine biochemical, mechanistic, and physiological effects of the substitutions in Japanese and northern bobwhite quail Mb on their three-dimensional structures compared to other Galliformes and avian species. Because of the emerging economic importance of Japanese quail in the poultry industry, further study can determine species-specific differences in color and color stability compared to other poultry species.

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Sequence  No.          10        20        30        40        50
J.Quail       GLSDQEWQQVLTIWGKVEADIAGHGHEVLMRLFRDHPETLDRFEKFKGLK
N.Bobwhite    GLSDQEWQQVLSIWGKVEADIAGHGHEVLMRLFRDHPETLDRFEKFKGLK
Chicken       GLSDQEWQQVLTIWGKVEADIAGHGHEVLMRLFHDHPETLDRFDKFKGLK
Duck          GLSDQEWQQVLTIWGKVEADLAGHGHAVLMRLFQDHPETLDRFEKFKGLK
Ostrich       GLSDQEWQQVLTIWGKVESDIAGHGHAILMRLFQDHPETLDRFEKFKGLT
Penguin       GLNDQEWQQVLTMWGKVESDLAGHGHAVLMRLFQDHPETMDRFDKFRGLK
Pig           GLSDGEWQLVLNVWGKVEADVAGHGQEVLIRLFKGHPETLEKFDKFKHLK
Horse         GLSDGEWQQVLNVWGKVEADIAGHGQEVLIRLFTGHPETLEKFDKFKHLK
Bovine        GLSDGEWQLVLNAWGKVEADVAGHGQEVLIRLFTGHPETLEKFDKFKHLK
Sheep         GLSDGEWQLVLNAWGKVEADVAGHGQEVLIRLFTGHPETLEKFDKFKHLK
Goat          GLSDGEWTLVLNAWGKVEADVAGHGQEVLIRLFTGHPETLEKFDKFKHLK

Sequence  No.          60        70        80        90       100
J.Quail       TPDQMKGSEDLKKHGATVLTQLGKILKQKGNHESELKPLAQTHATKHKIP
N.Bobwhite    TPDQMKGSEDLKKHGATVLTQLGKILKQKGNHESELKPLAQTHATKHKIP
Chicken       TPDQMKGSEDLKKHGATVLTQLGKILKQKGNHESELKPLAQTHATKHKIP
Duck          TPDQMKGSEDLKKHGVTVLTQLGKILKQKGNHEAELKPLAQTHATKHKIP
Ostrich       TPEQMKASEELKKHGVTVLTQLGKILKQKGKHEAELKPLAQTHATKHKIP
Penguin       TPDQMRGSEDMKKHGVTVLTQLGKILKQKGHHESELKPLAQTHATKHRVP
Pig           SEDEMKASEDLKKHGNTVLTALGGILKKKGHHEAELTPLAQSHATKHKIP
Horse         TEAEMKASEDLKKHGTVVLTALGGILKKKGHHEAELKPLAQSHATKHKIP
Bovine        TEAEMKASEDLKKHGNTVLTALGGILKKKGHHEAEVKHLAESHANKHKIP
Sheep         TEAEMKASEDLKKHGNTVLTALGGILKKKGHHEAEVKHLAESHANKHKIP
Goat          TGAEMKASEDLKKHGNTVLTALGGILKKKGHHEAEVKHLAESHANKHKIP

Sequence  No.         110       120       130       140       150
J.Quail       VKYLEFISEVIIKVIAEKHASDFGADSQAAMKKALELFRNDMASKYKEFGFQG
N.Bobwhite    VKYLEFISEVIIKVIAEKHAADFGADSQAAMKKALELFRNDMAAKYKEFGFQG
Chicken       VKYLEFISEVIIKVIAEKHAADFGADSQAAMKKALELFRNDMASKYKEFGFQG
Duck          VKYLEFISEVIIKVIAEKHSADFGADSQAAMKKALELFRNDMASKYKEFGFQG
Ostrich       VKYLEFISEVIIKVIAEKHSADFGADSQAAMKKALELFRNDMASKYKEFGFQG
Penguin       VKYLEFICEAIMKVIAEKHSADFGANCQAAMKKALELFRHDMASRYKEFGFQG
Pig           VKYLEFISEAIIQVLQSKHPGDFGADAQGAMSKALELFRNDMAAKYKELGFQG
Horse         IKYLEFISDAIIHVLHSKHPGDFGADAQGAMTKALELFRNDIAAKYKELGFQG
Bovine        VKYLEFISDAIIHVLHAKHPSDFGADAQAAMSKALELFRNDMAAQYKVLGFHG
Sheep         VKYLEFISDAIIHVLHAKHPSDFGADAQGAMSKALELFRNDMAAQYKVLGFQG
Goat          VKYLEFISDAIIHVLHAKHPSDFGADAQGAMSKALELFRNDMAAQYKVLGFQG
```

Figure 2.1 Amino acid sequence of Japanese quail and northern bobwhite myoglobin compared to other species. Differences between Japanese quail, northern bobwhite, and chicken at positions 12, 34, 44, 122, and 145 are shaded. J. Quail, *Coturnix japonica*; N. Bobwhite, *Colinus virginianus*; Chicken, *Gallus gallus*; Duck, *Anas platyrhynchos*; Ostrich, *Struthio camelus*; Penguin, *Aptenodytes forsteri*; Pig, *Sus scrofa*; Horse, *Equus ferus*; Bovine, *Bos taurus*; Sheep, *Ovis aries*; Goat, *Capra hircus*.

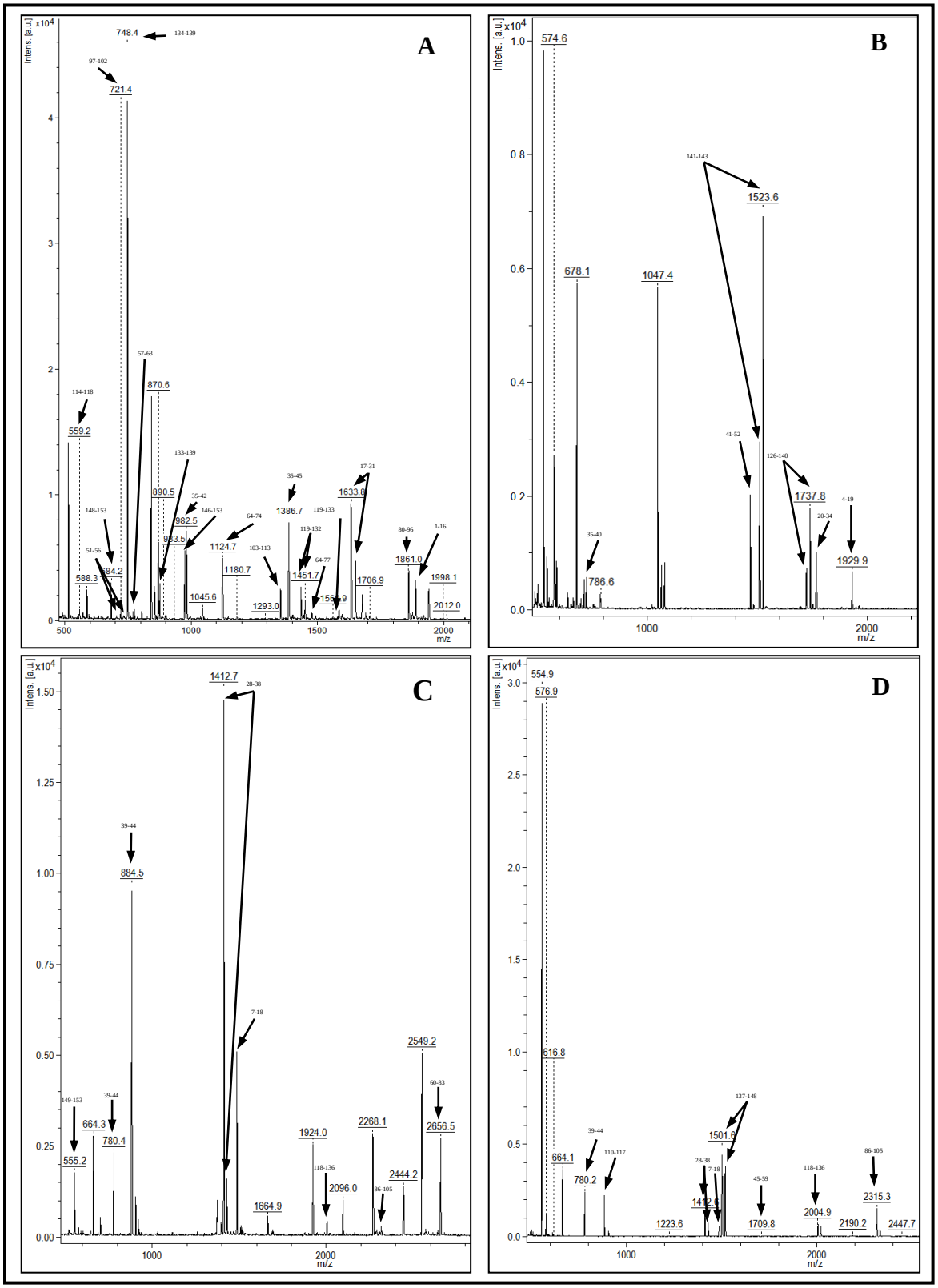

Figure 2.2 MALDI-TOF spectra of in-gel digests for Japanese quail Mb. Relevant peptides are labeled by arrows: A) Trypsin, B) Asp-N, C) Glu-C (100 mM Ammonium bicarbonate digest buffer) and D)Glu-C (25 mM Ammonium bicarbonate digest buffer).

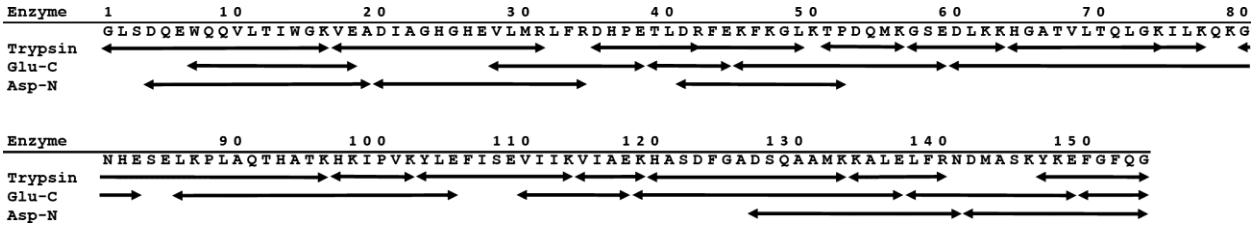

Figure 2.3 Sequence coverage of Japanese quail myoglobin by enzymatic digestion using endoproteinases Trypsin, Asp-N, and Glu-C. Double-sided arrows represent peptides detected by MALDI-TOF.

Table 2.1 Sequence of primers used for cDNA cloning

Primer	Sequence
JQF	5'-AACCATGGGGCTCAGCGACCA-3'
JQR	5'-GCATACATGAAGCCAGGAAAGG-3'
BWX1F	5'-AGGCAACAGCCGTAGGCAGCA-3'
BWX1R	5'-CTCATCAGAACCTCATGTCCAT-3'
BWX3F	5'-TCATTTCTGAAGTCAAGGTCAT-3'
BWX3R	5'-GGTGACAGATAACCCCTTATATTATTTT-3'
BWWF	5'-ACCATGGGGCTCAGCGACCA-3'
BWWR	5'-GGTGACAGATAACCCCTTATATTAT-3'

Table 2.2 Percentage sequence identity among Japanese quail and northern bobwhite myoglobin and other myoglobin.

Species	J. Quail	Chicken	Bobwhite	Duck	Ostrich	Penguin	Pig	Horse	Bovine	Sheep	Goat
J. Quail	100.0										
Chicken	98.04	100.0									
Bobwhite	98.04	97.39	100.0								
Duck	95.42	95.42	94.77	100.0							
Ostrich	91.50	91.50	90.85	94.77	100.0						
Penguin	84.31	85.62	83.66	87.58	84.31	100.0					
Pig	75.82	76.47	76.47	75.82	73.20	68.63	100.0				
Horse	75.16	75.82	75.82	74.51	73.20	67.32	90.85	100.0			
Bovine	72.55	72.55	72.55	72.55	69.93	65.36	88.24	88.24	100.0		
Sheep	72.55	72.55	72.55	71.90	69.93	65.36	89.54	89.54	98.69	100.0	
Goat	71.90	71.90	71.90	71.24	69.28	64.71	88.24	88.24	97.39	98.69	100.0

J. Quail, *Coturnix japonica*; Chicken, *Gallus gallus*; Bobwhite, *Colinus virginianus*; Duck, *Anas platyrhynchos*; Ostrich, *Struthio camelus*; Penguin, *Aptenodytes forsteri*; Pig, *Sus scrofa*; Horse, *Equus ferus*; Bovine, *Bos taurus*; Sheep, *Ovis aries*; Goat, *Capra hircus*

CHAPTER 3

CONCLUSIONS

- The amino acid sequences of Japanese quail and northern bobwhite Mb are comprised of 153 residues as presented in Fig 2.1.
- The calculated molecular weights from the deduced sequences of Japanese and northern bobwhite Mb, were 17,340 and 17,294 Da, respectively.
- The calculated isoelectric points of Japanese and northern bobwhite quail Mb are the same at 8.71, similar to duck and ostrich Mb.
- Japanese quail Mb has high sequence identity (98.0%) with chicken and northern bobwhite Mb, differing at only three residues in each.
- Northern bobwhite Mb has four substitutions and slightly less sequence identity (97.4%) with chicken Mb.
- Northern bobwhite and Japanese quail Mb both contain one less histidine than the nine present in chicken and turkey, substituting histidine 34 with arginine.