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Species-Specificity in Myoglobin Oxygenation and Reduction Potential Properties

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Abstract: The objective was to compare oxygenation and reduction potential properties of bovine and porcine myoglobins in-vitro. Cyclic voltammetry and homology-based myoglobin modeling were used to determine the species-specific effects on myoglobin reduction potential and oxygenation properties at pH 5.6, 6.4, and 7.4. At all pHs, porcine myoglobin had greater ($P = 0.04$) oxygen affinity than bovine myoglobin. For both species, oxygen affinity was higher at pH 6.4 > pH 7.4 > 5.6 ($P = 0.0002$). Myoglobin reduction potential for both species was affected by pH ($P < 0.0001$). The redox potentials became more negative as pH increased, indicating a proton-coupled electron transfer. There were no differences ($P = 0.51$) between species in reduction potential properties of heme. Homology-based myoglobin modeling indicated that the porcine myoglobin has a shorter distance between the distal histidine and heme than does bovine myoglobin. The variation in amino acid composition between bovine and porcine myoglobin could be partially responsible for differences in oxygen affinity.

Keywords: meat color, metmyoglobin, myoglobin, myoglobin reduction, oxygenation
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Introduction

The characteristic bright cherry-red color of beef is the primary determinant that influences consumers' decision to purchase meat (Mancini and Hunt, 2005). Myoglobin is the protein primarily responsible for meat color, and it can exist in 3 different forms; namely deoxy-, oxy-, or metmyoglobin. Myoglobin consists of a heme ring surrounded by globin. The iron present in the heme has 6 coordination sites, of which, 4 are occupied by protoporphyrin ring, fifth by His-93 residue, and the sixth site by ligands such as oxygen, water, or carbon monoxide. The ligand attached to the central heme and the valence state of iron determines meat color. In deoxymyoglobin, heme is in the ferrous state and no ligand is attached. Oxygen binding to ferrous heme results in the formation of oxymyoglobin. However, oxidation of oxy- and deoxymyoglobin leads to metmyoglobin formation (AMSA, 2012; Jayasingh et al., 2001). Hence, processes that can promote oxymyoglobin formation (oxygenation) and metmyoglobin reducing activity can influence meat color.

Meat has an inherent capacity to limit myoglobin oxidation by a process called metmyoglobin reducing activity. To date, research has been focused on the role of enzymes, mitochondria, and NADH levels in metmyoglobin reducing capacity (Lanari and Cassens, 1991; Tang et al., 2005; Kim et al., 2006). The ability of the heme to undergo reduction and/or oxygen binding ability and amino acid sequence also can affect meat color.

The application of electrochemical methods will help to investigate the changes in the microenvironment to the atomic level of heme and at the active site

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of myoglobin (Qian et al., 2013; Nerimetla et al., 2014). Thus, characterizing the interrelationship between myoglobin reduction potential and oxygen binding properties will increase our knowledge related to meat color.

Previous research reported that myoglobin oxidation is species-specific and it depends on the primary amino acid sequence (Yin et al., 2011). More specifically, the number of histidine residues in the myoglobin of meat-producing livestock species can influence myoglobin redox stability (Suman et al., 2007). The amino acid composition can affect the 3-dimensional structure, hence the ability of heme to accept an electron or oxygen. Nevertheless, we are not aware of any report detailing the species-specific effects on myoglobin reduction potential and oxygen binding capacity. Therefore, the objective of the current study was to compare the myoglobin oxygenation and reduction potential properties of bovine and porcine myoglobins using electrochemical methods *in-vitro*.

Materials and Methods

Bovine and porcine hearts were obtained from a USDA Inspected processing facility. Therefore, a review of procedures by Oklahoma State University Institutional Animal Care and Use Committee was not needed.

Materials and chemicals

High-purity graphite (HPG) disc electrodes (geometric area 0.2 cm^2) were purchased from McMaster-Carr (Atlanta, GA). Silicon carbide (SiC) paper, monosodium hydrogen phosphate ($NaH_2PO_4.H_2O$), disodium hydrogen phosphate (Na_2HPO_4) and sodium chloride (NaCl) were purchased from Sigma-Aldrich (St. Louis, MO). All chemicals were of analytical grade or greater purity.

Myoglobin isolation

Ammonium sulfate precipitation and gel-filtration techniques were used to isolate myoglobin from porcine and bovine heart (Faustman and Phillips, 2001). Briefly, beef and porcine cardiac muscle devoid of fat and connective tissue was homogenized in buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, 4°C) and centrifuged at 5,000 × *g* for 10 min. The supernatant was brought to 70% ammonium sulfate saturation, and the resulting solution was stirred for 1 h at 4°C and then centrifuged at 18,000 × *g* for 20 min. The supernatant was then saturated with ammonium sulfate (100%) and centrifuged at 20,000 × *g* for 1 h. The precipitate was resuspended in homogenization buffer and dialyzed (3 volumes) against 10 mM Tris-HCl, 1 mM EDTA, at pH 8.0, 4°C for 24 h. Myoglobin was separated from hemoglobin using a Sephacryl 200-HR gel filtration column (2.5 × 100 cm) using 5 mM Tris-HCl, 1 mM EDTA, pH 8.0 as the mobile phase at 1 mL/min. The isolated myoglobin was a mixture of oxy-, deoxy-, and metmyoglobin. To limit the interference of oxidizing agents on metmyoglobin reduction, myoglobin solution was transferred into a glass tube, and metmyoglobin was prepared by incubating myoglobin at 37°C for 24 h . Samples were stored at -80°C until use.

Electrochemical studies

Immobilization of beef and porcine Mb on high purity graphite electrodes. High purity graphite (HPG) electrodes were polished using a silicon carbide paper (P320 grit) and sonicated for 30 s. After rinsed in deionized water, the electrodes were dried under nitrogen. Ten microliters of purified bovine or porcine metmyoglobin (0.15 mM) were adsorbed on freshly polished HPG electrodes and incubated for 10 min at 4°C to allow physisorption involving electrostatic and other secondary interactions (Van Dyke et al., 1996). The electrodes were then rinsed in deionized water to remove unbound myoglobin and the myoglobin coated HPG electrodes were used for electrochemical studies.

Myoglobin reduction potential and oxygenation measurements. Myoglobin reduction potential and oxygenation properties were determined by the methodology described by Nerimetla et al. (2014). Cyclic voltammetry was used to determine reduction potential and oxygenation properties of bovine and porcine myoglobins. Cyclic voltammetry (CV) studies were performed using CH Instrument (CHI 1040, CH Instruments, Inc. Electrochemical Instrumentation, Austin, TX) with HPG as the working electrode, platinum (Pt) wire as a counter electrode, and Ag/AgCl (1 M KCl) as the reference electrode. The cyclic voltammetry experiments were performed using 0.1 M of 10 mL mixed phosphate buffer (pH 5.6, 6.4, and 7.4) under anaerobic conditions, and these pH conditions represented postmortem muscle, dark-cutting beef, and physiological pH, respectively. Different pH buffers were prepared by mixing mono- and disodium hydrogen phosphate. The cyclic voltammetry experiments were performed at room temperature.

For oxygenation studies, various concentrations of oxygen (0 to 1,200 μM) and nitrogen were supplied to the electrochemical cell using 2 mass flow controllers (Aalborg Instruments & Controls Inc., Orangeburg,

NY; model GFC17) and the percentage oxygen was converted to molar concentration using the Henry's equation (Loomis, 1928; Kavanaugh and Trussell, 1980). Nitrogen and oxygen gases were of ultra-high purity and source was from Matheson Tri-Gas (Basking Ridge, NJ). Zero percent oxygen level was achieved by purging nitrogen gas. Unless otherwise specified, all electrochemical measurements were performed in an anaerobic mixed phosphate buffer (0.1 M containing 0.15 M NaCl; pH 5.6, 6.4, and 7.4) under a nitrogen atmosphere. To investigate the oxymyoglobin formation constants at pH 5.6, 6.4, and 7.4, electrochemical Michaelis-Menten kinetics were employed (Guto and Rusling, 2005). The formation of an oxymyoglobin (Fe^{II}–O_2) complex on the reduction of metmyoglobin in the presence of oxygen gives rise to catalytic currents in proportion to oxygen concentration (due to the reduction of formed Fe^{II}–O_2 on the electrode surface at negative potential region). The greater the oxygen binding, the larger is the resulting current. The relationship between oxymyoglobin reduction current and concentration of oxygen fits well in the Michaelis-Menten equation. The following formula was used to determine the myoglobin affinity for oxygen.

$$I_{cat} = \frac{nFA\Gamma k_{cat} C_s}{C_s + K_M}$$

n is the number of electrons in the reaction (n = 2), F is faraday's constant, A is the electrode area (0.2 cm^2), Γ is the electroactive amount (mol cm^{-2}) of protein on an electrode, C_s is the concentration of oxygen, and K_M is the oxymyoglobin formation constant or Michaelis-Menten constant. K_M indicates the affinity of oxygen to bind with the reduced heme; a lower number indicates greater oxygen affinity. I_{cat} is the catalytic property of the enzyme when it reacts with the substrate. It increases when substrate (O_2) binds to myoglobin in its reduced state. k_{cat} is a catalytic constant also called as turnover number. Turnover number of an enzyme (myoglobin) is the number of substrate molecules (O_2) that are converted to product per unit time when the enzyme (myoglobin) is fully saturated with substrate (O_2).

A range of 0 to -0.6 V was applied to the myoglobin containing electrodes for reduction potential studies. The respective buffer was purged with nitrogen gas to attain zero oxygen concentration. Cyclic voltammetry was performed at different pH conditions to obtain reduction and oxidation peak potentials. The electrode was rotated (Eco Chemie Autolab rotator with a motor controller, Metrohm Inc., Utrecht, the Netherlands) at a rate of 2,000 rpm to get steady state conditions. A lower number indicates myoglobin has greater reduction potential or a tendency to get reduced quickly.

Myoglobin modeling

The structure of the bovine (*Bos taurus*) myoglobin was predicted using the structure of porcine oxy myoglobin form (protein data bank id: PDB 1 MNO), a homolog, for which the 3-dimensional structure has been determined by X-ray diffraction method (Krzywda et al., 1998). This homology-based modeling was conducted using the Iterative Threading Assembly Refinement (I-TASSER) server (Zhang, 2008; Roy et al., 2010; Yang et al., 2015; Yang and Zhang, 2015). This homology-based modeling study with I-TASSER involves 3 steps: template identification from PDB library, assembly of iterative structures, and structure-based function annotation. I-TASSER identifies the homologous structure models from PDB database (Dutta et al., 2007) using an algorithm Local Meta-Threading-Server (LOMETS; Wu and Zhang, 2007). The secondary structure of the target protein was predicted based on sequence information from the Protein Secondary Structure PREDiction (PSSpred) algorithm (Yang, Yan, Roy, Xu, Poisson & Zhang, 2015). The lowest free energy conformations were determined by SPICKER (a clustering approach to identify near-native protein folds; Zhang and Skolnick, 2004). Refinement of the low free energy conformations was done by using FG-MD (Fragment Guided Molecular Dynamics simulations; Zhang et al., 2011) and ModRefiner (Xu and Zhang, 2011). Prediction of the ligand-binding site of the target protein was made by COACH algorithm (Yang et al., 2013).

The distance between N_ε of the His64, His93, and His 97 and the iron and oxygen molecule of the heme group in the crystal structure of porcine myoglobin (PDB: 1MNO) was calculated using PyMOL (version 1.8, Schrödinger, LLC, New York, NY). The distance between N_ε of the His64, His93, and His 97 and the iron molecule of the heme group in the predicted structure of bovine myoglobin was also determined using PyMOL.

Statistical analysis

The experimental design was a completely randomized design (n = 6). The fixed effects include pH, species, and their interaction. Type-3 tests of fixed effects were performed using the MIXED Procedure of SAS (Version 9.1, SAS Inst. Inc., Cary, NC). Least squares

Table 1. Effects of pH and species on oxygenation property[1] on porcine and bovine myoglobins

Trait	Variable	Oxygen affinity (μM)[1]	SE	*P*-value
pH effect	pH 5.6	852.4	29.4	0.0002
	pH 6.4	591.2		
	pH 7.4	749.5		
Species effect	Beef	851.4	24.0	< 0.0001
	Pork	610.2		
pH × species effect	pH 5.6, beef	932.4[d]	41.2	0.0420
	pH 5.6, pork	772.4[bc]		
	pH 6.4, beef	778.4[c]		
	pH 6.4, pork	404.5[a]		
	pH 7.4, beef	844.0[cd]		
	pH 7.4, pork	655.0[b]		

[a-d]Least square means with different superscripts significantly differ ($P < 0.05$).

[1]Michaelis-Menten constants (K_M) indicate the oxygen affinity in micromolar. Lower K_M value indicates a greater affinity for oxygen binding to Mb at a given pH condition.

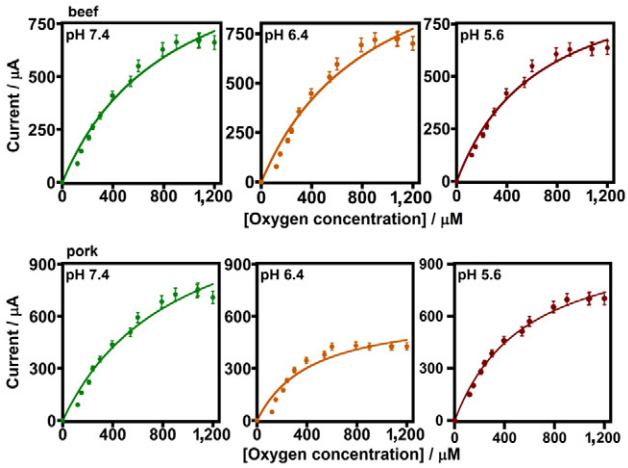

Figure 1. The Michaelis- Menten kinetics[1] observed for the reduction currents of beef and pork myoglobin versus O_2 concentration at pH 5.6, 6.4, and 7.4. [1]Y-axis represents the catalytic current (I_{cat}) (i. e, oxymyoglobin formation) corresponding to the concentration of oxygen on the X-axis. The injection of different concentrations of oxygen into the solution increased oxymyoglobin formation as indicated by increased current. The relationship between oxygen concentration and resultant current followed the Michaelis-Menten kinetics data fit in a K-graph. Kinetic parameters (K_M and k_{cat}) were extracted from Michaelis-Menten figure data.

Table 2. Comparison of distance (in Angstrom) from proximal and distance histidine to heme iron in bovine and porcine myoglobins

Distance measured	Bovine	Porcine
His64 N_ε- Fe	5.6	4.3
His64 N_ε- oxygen	**	2.6
His93 N_ε- Fe	2.2	2.2
His97 N_ε- Fe	5.5	5.4

**Bovine myoglobin has not been crystallized yet. Hence, the distance between His64 N_ε- oxygen was not calculated.

means for protected F-tests ($P < 0.05$) were separated by using the pdiff option (least significant differences) and were considered significant at $P < 0.05$.

Results

Effects of pH and species on oxygenation properties

For oxygenation studies, 0 to 1,600 μM of oxygen was allowed to bind with myoglobin at pH 5.6, 6.4, or 7.4. A pH × species interaction resulted for oxygenation properties ($P = 0.04$; Table 1). Porcine myoglobin had greater oxygen affinity than beef myoglobin at the same pH ($P = 0.04$; Fig. 1 and Table 1). The formation of oxymyoglobin results in a catalytic current. Figure 1 represents oxymyoglobin formation with different levels of oxygen. The relationship between oxygen concentration and resultant current followed the Michaelis-Menten kinetics data. The numbers in the Table 1 were derived from the Michaelis-Menten kinetics data using a K-graph software. Kinetic parameters (K_M and k_{cat}) were extracted from Michaelis-Menten figure data. A lower K_M value indicates a greater affinity for oxygen binding to myoglobin at a given pH condition. The homology-based myoglobin modeling indicates that the porcine myoglobin has a shorter distance between distal histidine and heme than bovine (Table 2 and Fig. 2).

Effects of pH and species on reduction potential properties

The main effect of pH was significant for myoglobin reduction potential ($P < 0.0001$; Table 3). At pH 5.6, myoglobin reduction potential was -306.3 mV compared with -362.8 mV at pH 7.4 ($P < 0.0001$). The reduction potential value provides information about the ability of heme within myoglobin to accept electrons. Myoglobin redox potentials shifted more negative with increasing pH from 5.6 to 7.4, indicating a proton-coupled electron transfer process. The reduction potential values demonstrated no differences between species ($P = 0.51$) and also for species × pH interaction ($P = 0.91$).

Discussion

Meat is a complex system; various processes and organelles such as mitochondria, lipid oxidation, and

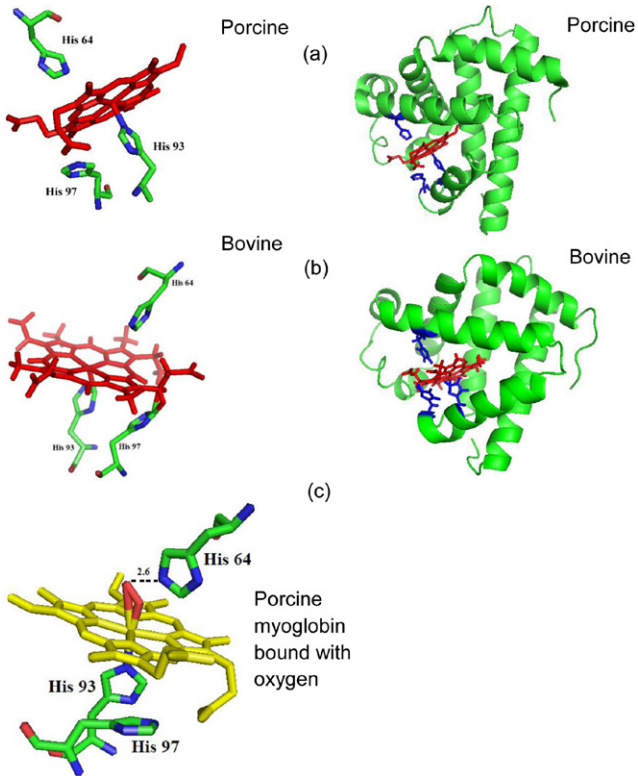

Figure 2. Three-dimensional representation of porcine and bovine myoglobins. The deoxy porcine myoglobin structure (Fig. 2a) was determined experimentally using X-ray crystallography (Krzywda et al., 1998). The homology-based modeling of bovine myoglobin (deoxy form) structure (Fig. 2b) was calculated based on porcine myoglobin structure (PDB# 1 MNO; Krzywda et al., 1998). Figure 2c is the oxy form of porcine myoglobin structure (PDB# 1 MNO, Krzywda et al., 1998).

Table 3. Effects of pH and species on reduction potential[1] properties on porcine and bovine myoglobins

Trait	Variable	Reduction potential (mV)	SE	*P*-value
pH effect	pH 5.6	-306.3[a]	2.3	< 0.0001
	pH 6.4	-319.2[b]		
	pH 7.4	-362.8[c]		
Species effect	Beef	-328.6	1.8	0.5162
	Pork	-330.3		
pH × species effect	pH 5.6, beef	-305.2	4.6	0.9185
	pH 5.6, pork	-307.4		
	pH 6.4, beef	-319.3		
	pH 6.4, pork	-319.0		
	pH 7.4, beef	-361.4		
	pH 7.4, pork	-364.5		

[a-c]Least square means with different superscripts significantly differ ($P < 0.05$).

[1]The reduction potential value provides information about the ability of heme within myoglobin to accept an electron. A lower number indicates that the myoglobin has a greater capacity for reduction.

microorganisms compete for oxygen. Hence, to study oxygen binding properties specifically to myoglobin in a meat system might be challenging. We utilized an electrochemical approach to understanding the biochemical basis of oxygen binding to myoglobin *in-vitro*. Various research has employed cyclic voltammetry to study protein functionality and successfully extrapolated the effects under physiological conditions (Guto and Rusling, 2005; Qian et al., 2013).

The mechanistic basis for species-specific effects of oxygen binding on myoglobin is not clear. In the current study, porcine myoglobin had greater oxygen affinity for each pH group than bovine myoglobin. Porcine and bovine myoglobin share 88% amino acid sequence homology; Fig. 3). Further, the differences in amino acid sequence between species could be partially responsible for changes in oxygen affinity. The amino acid sequence can affect the net charge of a protein and its affinity for oxygen. Marcinek et al. (2001) reported that the oxygen binding depends on the amino acid sequence surrounding the heme pocket. In addition, the primary amino acid sequence can affect the tertiary structure, which in turn can influence interaction with ligands and also the volume of the heme cavity (Suman et al., 2007). In support, Bunn (1971) reported that porcine hemoglobin has greater oxygen affinity than bovine hemoglobin, due to differences in amino acid composition. Furthermore, variation in the primary structure was responsible for species-specific effects of secondary lipid oxidation products on myoglobin redox stability (Suman et al., 2007). For example, porcine myoglobin contains 9 histidine residues, while bovine myoglobin contains 13 residues. The lower the number of nucleophilic histidine in porcine myoglobin than bovine is responsible for its greater redox stability against 4-hydroxy-2-nonenal. More specifically, 4-hydroxy-2-nonenal can preferentially bind with nucleophilic histidine that are available for reaction.

In addition to differences in the number of histidine residues, differences in distances between distal histidine and heme can influence oxygen binding. The distal histidine plays a significant role in stabilizing the oxygen bound to heme. A shorter distance between distal histidine and heme in porcine myoglobin might have favored oxygen binding to heme compared with bovine myoglobin.

At pH 5.6, the net charge of bovine myoglobin and porcine myoglobin were, 12.4 and 8.8, respectively (Protein Calculator v3.4, http://protcalc.sourceforge.net/). A greater positive charge could be less favorable for oxygen to bind with beef myoglobin. Although the exact mechanism is not clear, we can speculate from medical research. More specifically, hemoglobin at a

lower pH (greater positive charge) has a lower affinity for oxygen due to the Bohr effect (Benesch and Benesch, 1961). However, in myoglobin, a lower pH minimizes heme-apomyoglobin contact (La Mar et al., 1978). This support the current research that the oxygen affinity was lower at pH 5.6 compared with 6.4 and 7.4 within a species. Previous research using steaks reported greater oxygenation or bloom in pork than beef (Haas and Brazlter, 1965). Greater oxygen affinity to porcine myoglobin, in part, could be responsible for more bloom in pork than beef.

Porcine myoglobin has been crystallized, but, bovine myoglobin has not been crystallized yet (Cai et al., 2016). Hence, it was not possible to compare the amino acid sequence surrounding the heme cavity. Interestingly, in the current study, for both species oxygen affinity was greater at pH 6.4 than pH 5.6 and 7.4. Distal histidine plays a major role in stabilizing ligands such as oxygen. Histidine pKa is 6.1. Since pKa is close to pH 6.4 compared with pH 5.6 and 7.4, a lower charge difference might have favored oxygenation.

In the present study, there was no significant effect of species on reduction potential. The results suggest there were no differences between species at a particular pH to accept an electron. However, there was a significant effect of pH on the reduction potential. A pH of 7.4 favored reduction more than pH 6.4 and 5.6. Previous research using equine myoglobin also reported a greater metmyoglobin reduction at pH 7.4 (Nerimetla et al., 2014). Greater metmyoglobin reducing activity at an elevated pH meat, in part, can be attributed to the ability of heme to accept electrons quicker than normal pH. Metmyoglobin reduction changes with conditions and also depends on the type of system used. For example, Elroy et al. (2015) reported that porcine and bovine myoglobin have different non-enzymatic metmyoglobin reduction capacity. The enzymatic reaction involves 3-dimensional binding of co-factors and proteins, which aid in electron transfer to ferric heme. However, application of a potential to graphite electrode will move electrons directly to heme.

Conclusions

Meat matrix is composed of various biomolecules; hence studying myoglobin functional properties can be challenging. The current study suggests that cyclic voltammetry is a useful tool to characterize myoglobin reduction potential and oxygenation properties. Oxygen binding to myoglobin is species-specific. Further homology-based modeling helps to explain the species-specific variation in myoglobin properties. The results indicate that cyclic voltammetry can be a valuable tool to understand meat biochemical properties such as the effects of pH on bloom, effects of temperature on myoglobin reduction potential, and to study the role of lipid oxidation products on myoglobin redox stability.

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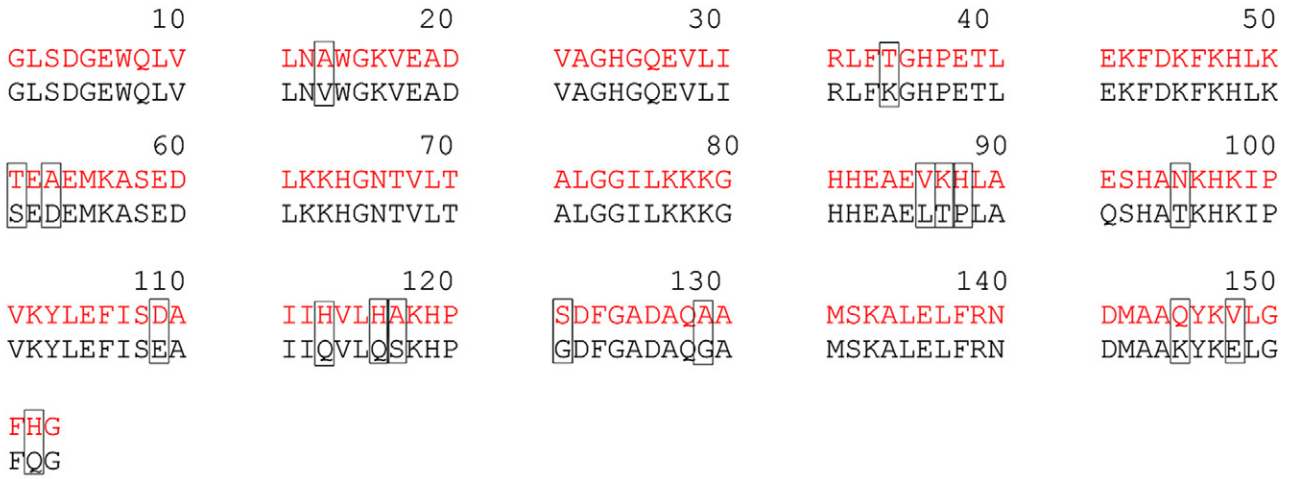

Figure 3. Comparison of amino acid sequences of porcine and bovine myoglobin. Differences in amino acids are represented by a box. The first row represents bovine myoglobin and the second row represents porcine myoglobin.

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