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Spectroscopic characterization of biochemical states of myoglobin in beef in different environments

Jae-Kwang Kim [a], Eun Kyoung Song [b], Tae Joo Park [b,*], Chang-Joon Kim [c,*]

[a] School of Energy & Chemical Engineering, Ulsan National Institute of Science and Technology (UNIST), Ulsan 689-798, Republic of Korea
[b] School of Life Science, Ulsan National Institute of Science and Technology (UNIST), Ulsan 689-798, Republic of Korea
[c] Department of Chemical Engineering & ERI, Gyeongsang National University, 501 Jinju-daero, Jinju, Gyeongnam 660-701, Republic of Korea

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ABSTRACT

The two states of myoglobin (Mb) in beef were firstly investigated using a spectrophotometer. Oxymyoglobin (Oxy-Mb) and metmyoglobin (Met-Mb) coexist in the primary beef, where the amount of each type determines the color of the beef. In this study, the influence of denaturing agents and pH on Mb was examined using fluorescence spectrometers to observe the behavior (folding and unfolding) of Mb as a function of different concentrations of denaturing agents (GuHCl and Urea) and different pH values. The unfolding of Mb is increased with an increasing concentration of denaturing agents. However, the unfolding decreases with an increase in pH, in accordance with its natural behavior.

Meat consumers often judge the quality of meat by its color. The color of meat mostly comes from myoglobin's heme group. Mammalian myoglobin (Mb) is a protein that binds oxygen in cardiac and skeletal muscle tissue. Mb is a monomeric protein of molecular weight 16.7 kDa [1]. Like hemoglobin, this protein binds oxygen with a prosthetic heme molecule, which consists of an iron atom covalently bound to the four nitrogen atoms of protoporphyrin IX. In oxygenated Mb, the iron forms two more bonds, one with oxygen and the other with proximal histidine residue [2,3].

Mb's primary function is to store the oxygen required by tissue. This protein has two stable forms: Fe^{3+}–H_2O-Mb (metmyoglobin) and Fe^{2+}–O_2-Mb (oxymyoglobin), as shown in Fig. 1. Oxymyoglobin (Oxy-Mb) gives a red color and fresh appearance to meat whereas metmyoglobin (Met-Mb) gives a brownish color to meat [4]. Characterization of the oxidation–reduction state of Mb in various meats in different environments is necessary in order to develop methods for storing meat while maintaining its freshness. The Mb conversion rates that depend on environmental conditions (pH, temperature, etc.) are unique to the different animals such as lysozyme [5–8].

The oxidizing and reducing reactions of Mb in living systems can be investigated in vitro using chemical agents. The Oxy-Mb of a functionally important state can be converted into inactive Met-Mb as the O_2 molecule is released and the H_2O molecule replaces it by the oxidation reaction in which an electron from a ferrous iron is donated to the oxidizing agent. Met-Mb can be converted into Oxy-Mb through a reduction reaction in which the heme iron gains an electron from the reducing agent.

Mb usually has a unique conformation in normal environments – this conformation enables it to function properly [9]. The presence of heat, detergents, pH extremes, high pressure, or chemical denaturants such as guanidine hydrochloride (GuHCl) and urea disrupts the structural organization of Mb [10–12] and results in unfolded protein. Especially, high denaturant concentrations can result in an almost complete unfolding of Mb into a random coil polypeptide chain, which can lead to the formation of aggregated protein masses [13]. Therefore, an exact understanding of the folding and unfolding reactions of Mb under different environmental conditions can provide invaluable clues to the development of methods to maintain meat freshness. This understanding can also be utilized to determine the optimal conditions in which to obtain a high amount of genetically expressed recombinant proteins in active soluble form [14].

The correlation between changes in the environment and Mb's ability to maintain its native folding enables prediction of the biological activity in a specific environment. Despite the importance of Mb in the meat and biotechnology industries, few analytical tools have been proposed to characterize the biochemical state of Mb.

* Corresponding authors.
E-mail addresses: parktj@unist.ac.kr (T.J. Park), cj_kim@gnu.ac.kr (C.-J. Kim).

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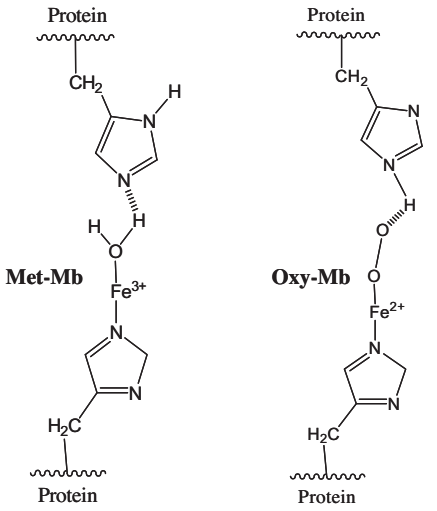

Fig. 1. Representation of the heme environment for Met-Mb (Fe^{3+}–H_2O-Mb) and Oxy-Mb (Fe^{2+}–O_2-Mb).

In this study, we suggested a simple and rapid analytical method to monitor the oxidation–reduction state or the structural organization of Mb using an ultraviolet visible spectrometer (UV–VIS) or a fluorometric spectrometer. Ultraviolet visible spectrometer (UV–VIS) and fluorometric spectrometer is useful analytical tools to interpret the biochemical properties [15,16]. UV–VIS analysis revealed the conversion between Oxy-Mb and Met-Mb through oxidizing or reducing agents. Using a fluorescence spectrometer, we monitored the extent of Mb denaturation at different concentrations of GuHCl or urea and different pH values.

Materials and methods

Oxidation and reduction of native myoglobin extracted from beef

Twenty milliliters of 0.1 M potassium phosphate buffer (pH 7.0) was added to a centrifuge tube containing 5 g of minced beef and was then mixed. Three separation layers were observed by centrifugation at 3780 × *g* at 6 °C for 45 min. The supernatant containing Mb was carefully removed without disturbing the pellet at the bottom or the fat in the top layer and was then transferred to a new tube. This was stored at 2–3 °C for later use. The reaction mixture, which had a total volume of 4 mL, contained 1 mL of Mb stock solution, 2 mL of 0.1 M phosphate buffer (pH 7.0), and 1 mL of 1 mM $K_6[FeCN_6]$ for oxidation or 1 mL of 1 mM $Na_2S_2O_4$ for the reduction of Mb. A native Mb solution (control) was also prepared by adding an additional 1 mL of phosphate buffer instead of oxidizing/reducing agents for comparison.

Denaturation of myoglobin

Stock solutions of guanidine hydrochloride (GuHCl) were prepared by dissolving accurately weighted GuHCl in 0.1 M potassium phosphate buffer (pH 7.0) to give a final concentration of 6.2 M. This was used for preparing a reaction mixture (3.9 mL) containing 0.2 mg/mL of Mb and GuHCl ranging from 0 M to 5 M. A 12 M urea stock solution was also prepared; it was used for preparing a reaction mixture containing 0.2 mg/mL of Mb and urea ranging from 0 M to 10 M. Phosphate buffer solutions of different pHs were prepared by mixing different volumes of 1 M solutions of monobasic potassium phosphate (KH_2PO_2, Aldrich) and dibasic potassium phosphate (K_2HPO_2, Aldrich). A 0.1 mL Mb solution in a phosphate buffer of concentration 0.15 mM was added to 5 mL of buffer solutions with different pH values.

Spectrometric analysis

UV–VIS absorptions of oxidized or reduced Mb were investigated in the range from 300 to 700 nm using a UV-VIS spectrophotometer (Cary 500, Varian, Inc., USA). The conformational changes of the protein could be determined by measuring the absorbance at 411 nm. The native (folding) or denatured (unfolded) states of Mb in samples were analyzed using a fluorescence spectrometer (SLM-Aminco Model 8100, Aminco Inc., USA). The fluorescence intensities were measured using an excitation wavelength of 285 nm and an emission wavelength of 340 nm. Intensities were corrected for instrument response. The fluorescence of the 0.1 M phosphate buffer solution was used for background correction by eliminating the Raman line of water (317 nm). The fraction of Mb unfolded (denatured), f_u, was determined by the following equation:

$$f_u = \frac{(I_n - I)}{(I_n - I_u)}$$

where I is the measured fluorescence intensity observed at the given GuHCl or urea concentration, I_n is the fluorescence intensity of the native state at ~320 nm, and I_u is the fluorescence intensity of the fully unfolded state at ~340 nm.

Results and discussion

The change in the structure of Mb by oxidation or reduction was investigated through analysis of the UV–VIS absorption spectra. Mb contains a porphyrin ring with an iron center. The 411 nm peak represents the porphyrin ring and the 500 nm peak depicts the hydrogen bonding with the dioxygen moiety [17]. The absorption spectra of native Mb solution (control), extracted from primary beef, have peaks appearing at 411, 500, 542, 581.5, and 635 nm in Fig. 2(a). The distinct peaks at 581.5 and 542 nm indicate the presence of a high amount of Oxy-Mb whereas the 635 nm peak indicates a small amount of Met-Mb. In addition, the Soret band at the 411 nm peak is shifted from the expected value of 417 nm [2]. When Mb was oxidized by $K_6[FeCN_6]$, absorption peaks at 581.5 and 542 nm, representing Oxy-MB, disappeared and new peaks started to appear around 529 nm as a result of Met-Mb. Although a weak Oxy-Mb peak remained after reaction with a small amount of oxidizing agent ($K_6[FeCN_6]$), the peaks at 635 and 529 nm were dominant after reaction with a larger amount, as shown in Fig. 2(b). This result clearly indicates that most Oxy-Mb can be converted to Met-Mb during oxidation by $K_6[FeCN_6]$. Spectrophotometric analysis can monitor this change.

The change in absorption intensity of the peak at 411 nm provides some information on the change in the folding or unfolding of Mb. If the peak intensity of a sample is higher than that of others, the Mb in that sample is more highly folded [18]. The completely oxidized sample (mostly Met-Mb, as shown in Fig. 2(b)) exhibits lower intensity than the native sample (mostly Oxy-Mb) (Fig. 2(a)). This indicates that Met-Mb is less folded than Oxy-Mb. This is probably because Met-Mb has weaker resistivity upon changing to unfolding than Oxy-Mb. In other words, Oxy-Mb can maintain the native folding better than Met-Mb. This result corresponds well with the fluorescence emission spectral data, as shown in Fig. 3. This behavior is also found in systems to store proteins at low temperatures and in Mb's solubility in ethylene glycol [18,19].

When the reducing agent ($Na_2S_2O_4$) was added to the oxidized sample, the intensity of the peak at 411 nm recovered to the level of the control sample and spectral peaks appeared at 542 and 581