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ARTICLE

Kinetics of Myoglobin Redox Form Stabilization by Malate Dehydrogenase

Anand Mohan,[†] S. Muthukrishnan,[‡] Melvin C. Hunt,*[,†]
Thomas J. Barstow,[§] and Terry A. Houser[†]

[†]Department of Animal Sciences and Industry, Weber Hall, Kansas State University, 224 Weber Hall, Manhattan, Kansas 66506, [‡]Department of Biochemistry, Chalmers Hall, Kansas State University, Manhattan, Kansas 66506, and [§]Department of Kinesiology, Kansas State University, Manhattan, Kansas 66506

This study reports the reduction of metmyoglobin (MMb) via oxidation of malate to oxaloacetate and the regeneration of reduced nicotinamide adenine dinucleotide (NADH) via malate dehydrogenase (MDH). Two experiments were conducted to evaluate a malate−MDH−NADH system as a possible mechanism for MMb reduction. In experiment 1, kinetics of MDH and MMb reduction were determined, and the results showed that increasing concentrations of oxidized nicotinamide adenine dinucleotide (NAD^+) and L-malate also increased ($p < 0.05$) MMb reduction in vitro. Experiment 2 assessed the reducing activity of beef muscle extracts with different concentrations of malate and NAD^+ added. Reduction of MMb in the muscle extracts via MDH was NAD^+, malate, and extract concentration dependent ($p < 0.05$). A new mechanism is described for the nonspecific and specific enzymatic reduction of MMb, which supports the hypothesis that malate can replenish NADH via MDH activity in post-mortem muscle, ultimately resulting in a more functional meat color.

KEYWORDS: Myoglobin; malate; meat color; MRA; MDH; NADH

INTRODUCTION

Meat color is primarily governed by the concentration and redox states of myoglobin present in meat. Changes in myoglobin redox chemistry and its effects on meat color play a vital role in muscle food appearance. Use of biobased ingredients that mediate post-mortem biochemical pathways affecting metmyoglobin-reducing activity (MRA) may provide new avenues to stabilize meat color (*1*). Recently, enzymatic systems that involve regeneration of the post-mortem pool of reduced nicotinamide dinucleotide (NADH) have been reported as highly effective pathways for metmyoglobin (MMb) reduction and meat color stabilization (*2*, *3*).

In meat, conversion of MMb to deoxymyoglobin (DMb) requires a reduction of oxygen partial pressure and MRA that uses NADH (*4*). Using endogenous enzyme systems that use numerous ingredients as metabolites for NADH regeneration has potential for adding value, extending product shelf life, and stabilizing meat color. Andrews et al. (*5*) noted that enzymes involved in glycolysis, the tricarboxylic acid cycle, and the electron transport chain remain active in post-mortem muscle and are capable of producing reducing equivalents. Watts et al. (*6*) and Saleh and Watts (*7*) suggested a reductive pathway through which intermediates of the glycolytic and tricarboxylic acid cycle pathways can be used for the production of NADH that is eventually used for MMb reduction. In a similar study, a specific MMb-reducing enzyme that required NADH for its activity was purified from bovine heart (*8*).

Mitochondria retain metabolic activity in post-mortem muscle for a long period of time and can consume oxygen and reduce MMb via enzymes located in the organelle (*9*). Several researchers (*10*−*12*) have suggested that meat discoloration is more indicative of muscle mitochondrial content and enzyme activity than other factors. Muscles with more mitochondria were associated with high oxidative metabolism and low color stability (*10*). Lanari and Cassens (*12*) reported that mitochondrial and submitochondrial particles facilitated myoglobin redox stability. Giddings (*13*) hypothesized that mitochondria may facilitate MMb reduction by supplementing meat tissue with a post-mortem pool of reduced cofactors (NADH) generated by reversal of the electron transport chain. Tang et al. (*14*) suggested that enzymes responsible for MMb reduction are located within the muscle's mitochondria and that the mitochondrial pool of NADH should provide for MRA. However, it is unknown how such enzymes within muscle mitochondria may relate to MRA.

NADH regenerated in the cytoplasm can be transferred to the mitochondria through the NADH shuttle for further use in the electron transport chain, which, in turn, helps transport metabolites between mitochondrial and cytosolic compartments. Moreover, numerous cytoplasmic processes will compete and use NADH, thus limiting the amount available for MMb reduction. Regeneration of the post-mortem pool of NADH by supplementing the tricarboxylic acid cycle enzyme, specifically malate dehydrogenase (MDH; L-malate: NAD^+ oxidoreductase, EC

*To whom correspondence should be addressed. Phone: +1 785 532 1232. Fax: +1 785 532 7059. E-mail address: hhunt@ksu.edu.

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Table 1. Nonspecific Enzymatic Reduction of Horse Metmyoglobin with Malate−MDH System at 30 °C and pH 5.7 (n = 3)

solution components[a]									
FMN 2.0 mM	MB 0.1 mM	NAD^+ 25 mM	NADH 2.0 mM	L-malic acid 100 mM	MDH[b] 0.1 mL	HMA 100 mM	GOT 0.1 mL	glutamate 100 mM	activity[c] (nmol/min)
+	+	+	−	+	+	−	+	+	1.59 ± 0.006
+	+	−	+	+	+	−	+	+	1.33 ± 0.013
−	+	+	−	+	+	−	+	+	1.26 ± 0.011
+	−	+	−	+	+	−	+	+	0.12 ± 0.005
+	+	−	−	+	+	−	+	+	0.01 ± 0.003
+	+	+	−	−	+	−	+	+	0.03 ± 0.007
+	+	+	−	+	−	−	+	+	0.02 ± 0.003
+	+	+	−	+	+	+	+	+	0.91 ± 0.004
+	+	+	−	+	+	−	−	+	0.23 ± 0.009
+	+	+	−	+	+	−	+	−	0.03 ± 0.003

[a] Substances present (+) or absent (−) in mixtures. FMN, flavin mononucleotide; HMA, hydroxymalonic acid; GOT, glutamate-oxaloacetate transaminase; MB, methylene blue; NAD^+, oxidized nicotinamide dinucleuotide; NADH, reduced nicotinamide dinucleuotide. Assays were conducted in an aerobic environment with 0.5 mM equine MMb in 40 mM phosphate buffer. Total assay volume (1.1 mL) composed of 0.2 mL of equine MMb and combinations of 0.1 mL of FMN, MB, NAD^+, l-malic acid, and hydroxymalonic acid with deionized distilled water to bring to final volume. [b] 226.8 units/mL. [c] Expressed as nonspecific MMb reducing activity.

1.1.1.37), with depleted substrate such as sodium malate in meat has not been reported. In this study, we investigated the role of MDH, a key member of the malate−aspartate shuttle, for its kinetics in NADH replenishment and the concomitant MMb reduction. Specific objectives were to (1) examine the interaction of malate and MDH to reduce MMb in vitro, (2) determine the influence of pH, temperature, concentrations of NAD^+ and malate on MDH enzyme activity and MMb reduction, and (3) determine the effects of malate on NADH generation, MRA, and color stability using beef muscle extracts.

MATERIALS AND METHODS

Experiment 1: Malate−MDH System in Nonspecific Enzymatic Reduction of Horse MMb. *Reagents*. Horse heart MMb, NAD^+, ethylenediaminetetraacetic acid (EDTA-disodium salt), methylene blue, L-malic acid, glutamate-oxaloacetate transaminase (GOT, from bovine heart muscle, 700 units/mg of protein), L-glutamic acid, and L-malate dehydrogenase (from bovine heart muscle, 600 units/mg of protein) were obtained from Sigma (St. Louis, MO). Water was purified through a Millipore-Q-Plus (Millipore, Amsterdam).

Assay Procedures. The roles of malate and MDH in nonspecific enzymatic reduction of horse MMb were assessed by adding various combinations of reactants to cuvettes (10 mm) containing 1.1 mL final reaction volume under aerobic conditions at 30 °C. The standard reaction mixtures at pH 5.7 (near normal pH for postrigor meat) contained one or more of the following (**Table 1**): 0.1 mL of 100 mM L-glutamic acid, 0.2 mL of 0.5 mM equine MMb in 40 mM phosphate buffer, 0.1 mL of citrate buffer (50, 80, 100, or 150 mM was used to adjust the pH of the assay buffer), 0.2 mL of 25 mM NAD^+, 0.1 mL of 100 mM L-malic acid, 0.1 mL of 0.1 mM methylene blue, 0.1 mL of GOT, and distilled deionized water to make the total reaction volume of the assay 1.0 mL. The reaction was initiated by adding 0.1 mL of MDH to the mixture (one unit reduced 1.0 μmol/min of malate to oxaloacetate). To measure MMb reduction, absorbance at 580 nm was recorded every 2 s for 5 min in a spectrophotometer (UV-2010; Hitachi Instruments, Inc., San Jose, CA). Nonspecific enzymatic reducing activity was calculated as nanomoles of MMb reduced, or nanomoles of oxymyoglobin (OMb) formed per minute during the initial linear phase of the assay using the difference in molar absorptivity of 12000 mol^{-1} cm^{-1} at 580 nm (the wavelength at which the difference in absorption between MMb and OMb is maximal). Activity was expressed as the mean of triplicate samples. Standard K_m analysis of MDH for requirements of cofactor NAD^+ and substrate malate was determined for different concentrations of NAD^+ and malate. The Michaelis constant (K_m) and maximum velocity (V_{max}) of the MDH for NAD^+ and malate were determined. To calculate K_m and V_{max}, for NAD^+ and malate, the Michaelis−Menten equation was converted to a linear form by taking the reciprocal of both sides of the equation. Initial velocity data were analyzed for K_m and V_{max} using the Lineweaver−Burk equation of:

$$\frac{1}{v_o} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$$

where K_m is the Michaelis constant of uninhibited MDH. This is the substrate concentration at which the velocity of the reaction is half the maximum velocity (V_{max})

Effects of pH. Effects of pH on the rate of nonspecific enzymatic reduction of horse MMb were assessed while keeping reactant concentrations constant. The final assay pH (5.7, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, or 10.0) was varied by altering the concentration of the mono- and disodium phosphate buffer by using a citrate buffer for the lower pHs or a tris/HCl buffer to adjust higher pHs. Nonspecific enzymatic reducing activity at different assay pH levels was calculated as nanomoles of MMb reduced (equal to nanomoles of OMb formed) per minute during the initial linear phase of the assay. Activity was expressed as the mean of triplicate samples.

Effects of NAD^+ Concentration. Rates of MMb reduction were determined at various NAD^+ concentrations at an assay pH of 5.7. Reduction of MMb was measured at NAD^+ concentrations of 2, 4, 6, 8, 10, 20, 40, and 80 mM containing 100 mM L-malic acid in 40 mM phosphate buffer. The absorbance changes at 580 nm were recorded every 2 s for 5 min in a spectrophotometer, and nonspecific enzymatic reducing activity was expressed as nanomoles of MMb reduced per minute during the initial linear phase of the assay. Activity was expressed as the mean of triplicate samples.

Effects of L-Malic Acid Concentration. Rates of MMb reduction were determined at L-malic acid concentrations of 1, 2, 4, 10, 20, 25, 50, and 100 mM at a NAD^+ concentration of 25 mM and an assay pH of 5.7. Absorbance changes at 580 nm were recorded every 2 s for 5 min in a spectrophotometer, and nonspecific enzymatic reducing activity was expressed as nanomoles of MMb reduced per minute during the initial linear phase of the assay. Furthermore, 0.1 mL of 200 mM hydroxymalonic acid was added to the mixture to investigate potential inhibiting effects on MDH in the MMb-reducing system. Activity was expressed as the mean of triplicate samples.

Effects of Processing Temperature. Effects of processing temperature (5, 10, 15, 20, 25, and 30 °C) on MMb reduction rates were determined with 25 mM NAD^+ and 100 mM L-malic acid at pH 5.7. Tubes containing reaction mixtures were incubated at their respective temperatures for 5, 10, 15, 30, and 60 min and then placed in 80 °C water to stop the reaction. After tubes were cooled to 30 °C, samples were assayed (30 °C) to determine MMb reduction by observing absorbance change at 580 nm in a spectrophotometer. Activity was expressed as the mean of triplicate samples.

Experiment 2: Malate−MDH−NADH System in Specific Enzymatic Reduction of Horse MMb via MMb Reductase. *Metmyoglobin Reduction in Muscle Extracts of Three Bovine Muscles*. Extracts of skeletal muscle were obtained from frozen (−80 °C) beef *M. longissimus dorsi* (LD), *M. psoas major* (PM), and *M. semitendinosus* (ST) muscles as described by Reddy and Carpenter (1991) with slight modifications. Briefly, beef muscle (5 g) was minced and homogenized in 25 mL of phosphate buffer (2.0 mM, pH 7.0) by using a homogenizer (Kinematica Polytron benchtop model PT 3100; Brinkmann, Lucerne, Switzerland) for 2 to 3 min. The homogenate was centrifuged at 35000*g* for 30 min at 4 °C, and the supernatant was filtered through a 0.45 μm micropore membrane to remove the fat layer. Oxyhemoproteins were oxidized with an excess of $K_3Fe(CN)_6$, and the solution was dialyzed (10000 MW cutoff membrane) at 4 °C with three 1 L per h changes of phosphate buffer (2 mM, pH 7.0) to remove excess ferricyanide. The solution was centrifuged at 15000*g* for 20 min at 4 °C, and the supernatant volume was adjusted to 25 mL with phosphate buffer (2.0 mM, pH 7.0).

Metmyoglobin Reducing Activity. Muscle extracts were evaluated for MRA using methodology described by Reddy and Carpenter (*15*) by adding various combinations of reactants to cuvettes 10 mm pathlength containing 1.10 mL final reaction volume under aerobic conditions. The standard assay mixture contained 0.10 mL of 5 mM EDTA, 0.10 mL of 40 mM phosphate buffer at pH 7.0, 0.10 mL of 3 mM $K_4Fe(CN)_6$, 0.20 mL of 0.5 mM MMb in 40 mM phosphate buffer pH 7.0, 0.30 mL of bovine muscle extract, 0.10 mL of 25 mM NAD^+, 0.10 mL of 100 mM malate solution, and distilled deionized water to make the total reaction volume of the assay 1.10 mL. The EDTA, $K_4Fe(CN)_6$, NAD^+, and L-malate solutions were prepared in 40 mM phosphate buffer pH 7.0. The reaction was initiated by addition of NAD^+ and malate. One unit of MRA was defined as the quantity that would reduce one nanomole of MMb per minute per gram of muscle during the initial linear phase of the assay using a difference in molar absorptivity of 12000 L mol^{-1} cm^{-1} at 580 nm. Assays were conducted at 30 °C. Effects of different amounts of enzyme extract, NAD^+ concentration, malate concentration, and assay pH on MMb reduction rate were studied. The formation of NADH was measured as an increase in absorbance at 340 nm immediately after the addition of enzyme extract to NAD^+ and malate incubation buffer (*16*). Assays for each extract and each set of conditions were performed on the same day in triplicate.

Statistical Analysis. Statistical analyses were performed using the MIXED procedure of SAS (SAS Institute, Inc., Release 9.3, Cary, NC; 2007). *F*-test denominator degrees of freedom were estimated using the Satterthwaite adjustment. Least squares means for significant *F*-tests were separated using least significant differences ($p < 0.05$).

RESULTS

Experiment 1: Malate−MDH System in Nonspecific Enzymatic Reduction of Horse MMb. The malate−MDH system reduced MMb enzymatically (nonspecific) most effectively with NAD^+ (1.59), slightly less with NADH substituted for NAD^+ (1.33), and slightly less reduction of MMb when FMN was excluded (1.26, **Table 1**). Addition of hydroxymalonic acid, a known MDH inhibitor (*17*, *18*), to the reaction mixture caused some decline in MMb reduction. Exclusion of MB, L-malic acid, MDH, GOT, or glutamate resulted in the lowest rates of MMb reduction. Replacing L-malic acid with D-malic acid in the assay mixtures would decrease MMb reduction because of the selective interaction of MDH with L-malic acid (*17*).

Effects of pH. The nonspecific enzymatic formation of NADH was pH dependent (**Figure 1A**); NADH formation increased as pH of the malate−MDH system increased from 5.5 to 10 and then decreased rapidly from pH 10 to 11.

Effects of pH on nonspecific enzymatic reduction of MMb were examined in phosphate buffer at 30 °C. As the assay pH increased, nonspecific enzymatic reduction of MMb increased (**Figure 1B**). The malate−MDH requirement for the cofactor [NADH (the MB was added only for electron transfer)] to reduce

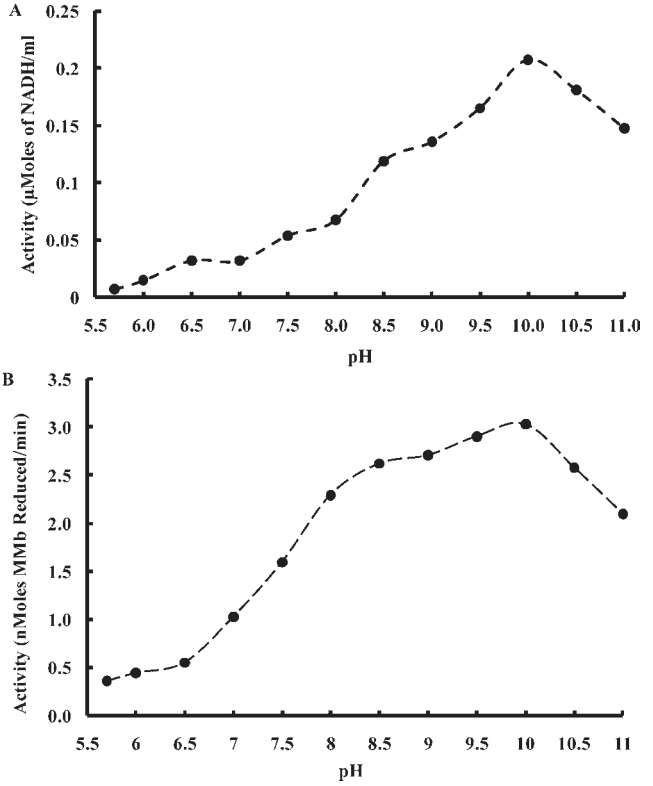

Figure 1. Effects of pH on (**A**) NADH production and (**B**) on initial velocity (v) of MMb reduction via a malate−MDH reaction pathway at 30 °C. Initial velocity is expressed as nanomoles of MMb reduced per minute; each point is the mean from four determinations. Standard error = 2.3.

MMb nonspecific enzymatically is probably a pH-dependent interaction. Nonspecific enzymatic reduction of MMb increased steadily to pH 8.5, increased more slowly to pH 10.0, and then declined to pH 11.0. Mild alkaline conditions appeared more conducive to MMb reduction in the malate−MDH model system.

Effects of NAD^+ Concentration. Increasing the amount of NAD^+ enhanced nonspecific enzymatic reduction of MMb at pH 5.7 and 30 °C (**Figure 2A**). Reduction of MMb proceeded linearly until malate was exhausted. The MMb reduction rate is apparently proportional to the rate of NADH formation and its concentration in the reaction mixture.

Effects of L-Malic Acid Concentration. **Figure 2B** shows the Lineweaver−Burk plot of MDH activity for MMb reduction using malic acid as a substrate. Increasing amounts of L-malic acid enhanced the nonspecific enzymatic MMb reduction rate at pH 5.7 and 30 °C (**Figure 2B**). Reduction of MMb in the malate−MDH reaction pathway proceeded linearly with increasing concentrations of malic acid. The K_m and V_{max} of MDH activity for MMb reduction were 1.1 mM and 2.54 nmol min^{-1}, respectively.

Effects of Assay Temperature. Reduction of MMb was slow (0.23 nMoles min^{-1}) at 5 °C but increased in a near-linear trend up to 30 °C (**Figure 3**). Because meat is generally stored at or below 5 °C, it appears that the malate−MDH system could slowly reduce MMb via NADH production.

Experiment 2: Malate−MDH System in Specific Enzymatic Reduction of Horse MMb via MMb Reductase. In this experiment, we observed specific enzymatic reduction of horse MMb by using muscle extracts from three bovine muscles (LD, PM, and ST) that are known to vary in relative color stability and muscle reductase.

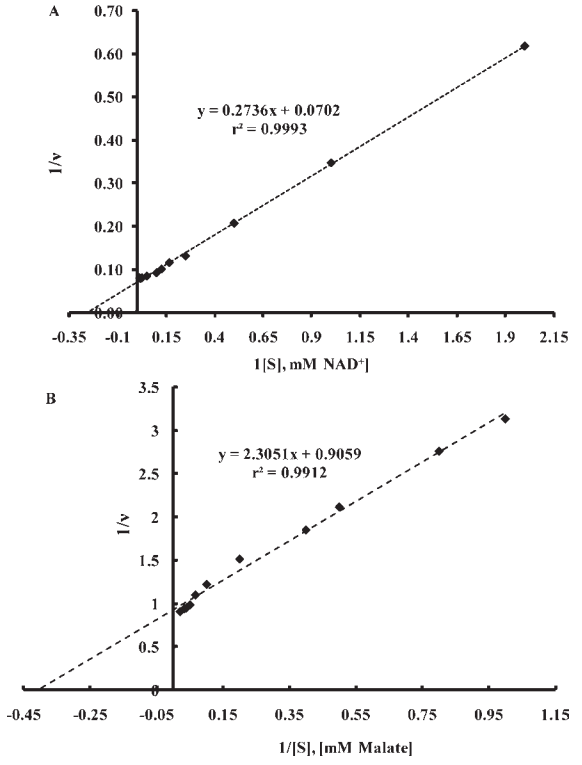

Figure 2. Effects of NAD^+ (**A**) and malate (**B**) concentration on initial velocity (v) of MMb reduction via a malate−MDH reaction pathway at pH 5.7 and 30 °C. Initial velocity is expressed as nanomoles of MMb reduced per minute; each point is the mean from four determinations.

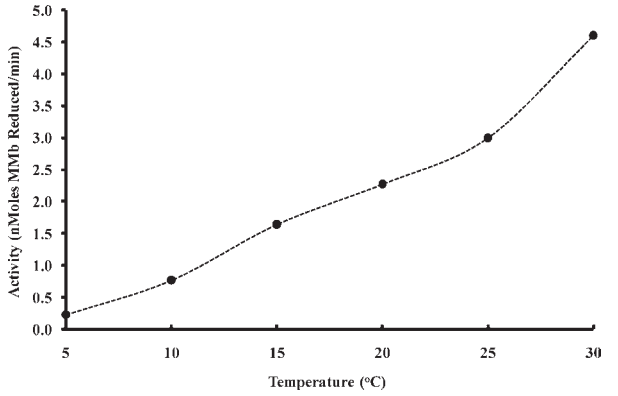

Figure 3. Effect of assay temperature on nonspecific enzymatic reduction of MMb via a malate−MDH reaction pathway at pH 5.7. Activity is expressed as nanomoles of MMb reduced per minute; each point is the mean from four determinations. Standard error = 1.8.

Regeneration of NADH via the malate−MDH system is shown in **Figure 4A**. Addition of NAD^+ and malate to the muscle extracts resulted in different rates of NADH for the three muscles. The NADH regeneration rate was highest for PM (10.1 nmol min^{-1} g^{-1}), intermediate for LD (7.6 nmol/min/g), and lowest for ST (5.8 nmol/min/g). Exclusion of malate and NAD^+ from the reaction mixture resulted in no NADH production. Additionally, substitution of malate with water in the reaction mixture containing NAD^+ produced NADH but at a very low rate. In the absence of malate, muscle extracts of the three beef muscles followed a trend similar to that observed for malate and NADH regeneration.

In a similar experiment, effects of NAD^+ reduction with concomitant formation of NADH were used to characterize

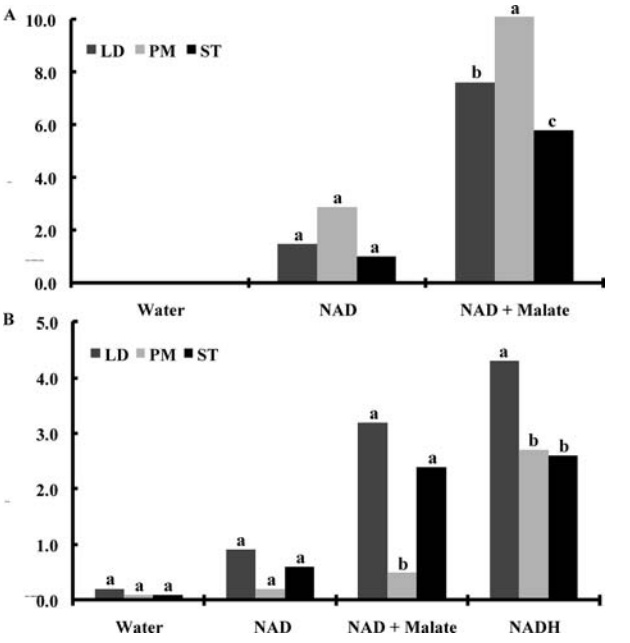

Figure 4. Production of NADH (**A**) and MRA (**B**) in muscle extracts from three beef muscles (longissimus dorsi [LD], psoas major [PM] and semitendinosus [ST]) with and without malate and a comparison between supplemented NADH and NADH produced via MDH. Standard error = 1.3 to 1.6. Bars within a trait with a different letter differ $p < 0.05$.

muscle color stability following MMb reduction. The malate−MDH system interacted with the bovine skeletal muscle MMb reductase enzyme to reduce horse MMb. Low MRA (LD, 0.9; ST, 0.6; and PM, 0.5 nmol min^{-1} g^{-1}) was observed in the absence of malate in the assay mixture (**Figure 4B**). Exclusion of malate in the muscle extract from the most color-stable muscle (LD) resulted in the highest MRA (0.9 nmol min^{-1} g^{-1}), ST (intermediate color stability) had intermediate MRA (0.6 nmol min^{-1} g^{-1}), and PM (lowest color stability) had the lowest MRA (0.5 nmol min^{-1} g^{-1}).

Addition of malate in the reaction mixture containing 0.2 mL of beef muscle extract from the different bovine muscles increased the MMb reduction rate in all three muscles ($p < 0.05$; LD, 3.2; ST, 2.4; and PM, 1.5 nmol MMb reduced min^{-1} g^{-1}, respectively). The MRA of the three beef muscles exhibited significant differences ($p < 0.05$) on MMb reduction rates when malate was added to muscle extracts (**Figure 4B**). The LD and ST had higher ($p < 0.05$) MRA than the PM, which had the lowest reducing activity. A similar trend was observed in the extracts from the three bovine muscles when standard NADH was added to the reaction. All muscles had increased rates of MMb reduction and compared with the LD; extracts from ST and PM showed no differences MMb reduction rates ($p > 0.05$; ST, 2.6; and PM, 2.7 nmol of MMb reduced min^{-1} g^{-1}).

Increasing the volume (0.02−0.2 mL) of muscle extracts from the three bovine muscles increased MMb reduction (**Figure 5A**) rate in a linear fashion ($p < 0.05$). For a given volume of extract, MMb reduction rate was greater for the most color-stable muscle (LD) than for the ST and PM muscles. Addition of more NAD^+ and L-malate to the standard reaction mixture also increased initial MMb reduction rates (**Figure 5B**,**C**). For the initial MMb reduction rate, the effect of NAD^+ concentration showed saturation kinetics, and the rate became independent of NAD^+ concentration above 6 mM L^{-1} (**Figure 5B**). For standard experimental conditions (0.2 mL of muscle extract from LD, ST, and PM, 0.5 mM MMb, pH 5.7), the Michaelis−Menten

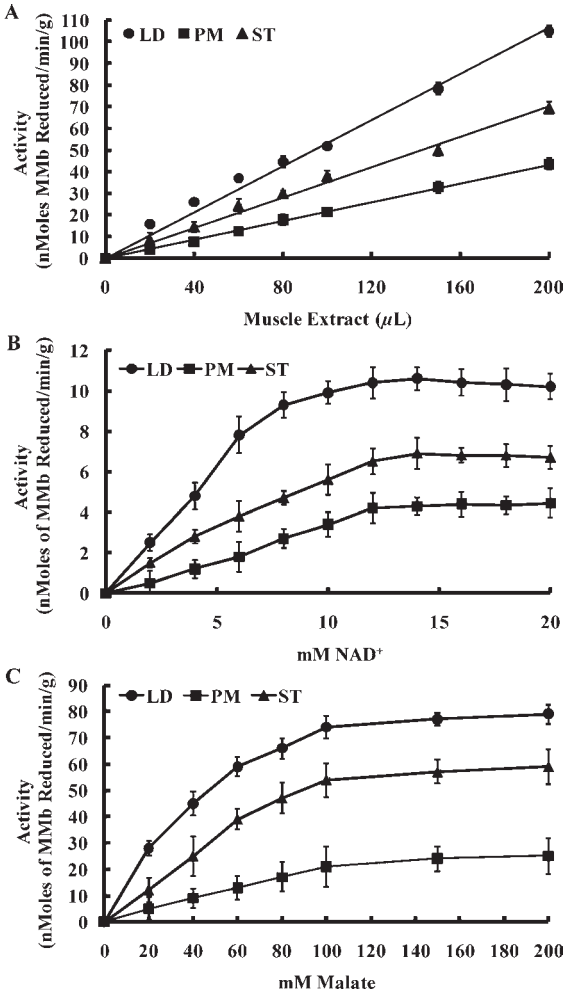

Figure 5. Concentration effects of the amount of muscle extract obtained from three beef muscles (longissimus dorsi [LD], psoas major [PM], and semitendinosus [ST]) (**A**), NAD^+ (**B**), and malate (**C**) on MRA of the solutions.

constant had a K_m value of 3.3×10^{-6} mol L^{-1}. However, initial velocity of the three muscles was different (LD, 4.8; ST, 2.7; and PM, 1.2×10^{-9} mol g^{-1} min^{-1}, respectively).

The enzymatic conditions for MMb reduction are limited not only by the amount of cofactors but also depended largely on the amount of substrate required for that enzymatic reaction to proceed. Similar behavior was observed with muscle extracts from three bovine muscles of varied color stability. Enzymatic reduction rates of MMb also were dependent on L-malate concentration. Addition of L-malate increased MMb reduction rate in the muscle extract from bovine muscles (**Figure 5C**). Of the three muscles, MMb reduction rate was highest in LD, intermediate in ST, and lowest in PM. For the initial MMb reduction rate, L-malate concentration showed saturation kinetics, and MMb reduction rate seemed to be independent of L-malate concentration above 80 mmol L^{-1}. For standard experimental conditions (0.2 mL of extract from LD, ST, and PM, 0.5 mM MMb, pH 7.5), the Michaelis–Menten constant had a K_m value of 42.2×10^{-6} mol·L^{-1}.

DISCUSSION

Reduction of Metmyoglobin in Vitro by Malate–MDH Activity. Saleh and Watts (*23*) described MMb reduction with the addition of malate. In this report, we report for the first time a potential detailed mechanism of nonspecific and specific enzymatic reduction of MMb via a malate–MDH system. A linear increase in MMb reduction with increasing concentrations of NAD^+ and malate suggests that MDH requires a cofactor and specificity toward the substrate malate to carry the enzymatic functions and a concomitant reduction of MMb in the malate–MDH system. Kim et al. (*19*) demonstrated that nonspecific enzymatic reduction of MMb in the lactate–LDH system was NAD^+ concentration dependent.

There are many commercial applications of natural enzymes in the meat industry in which properties of natural enzyme catalysis improve the quality characteristics of muscle foods. We investigated the potential of an oxidoreductase enzyme, MDH, to perform a particular biological role (i.e., reduction of NAD^+) to regenerate a post-mortem pool of NADH as an application for improving meat color and color stability. Mitochondrial MDH catalyzes the interconversion of malate and oxaloacetate linked to the oxidation/reduction of dinucleotide coenzymes, whereas cytoplasmic MDH is responsible for shuttling NADH across the mitochondrial membrane via the malate–aspartate shuttle (*20*). This study represents the first investigation of the role of a malate–NAD–MDH system in postrigor bovine muscle for MMb reduction *in vitro* and its ability to enhance MRA, which may involve both cytoplasmic and mitochondrial components. The concept of compartmentalization of reducing components is further investigated by Mohan et al. (*21*).

As shown in **Table 1**, nonspecific enzymatic reduction of MMb via malate–MDH is an efficient source of regenerating reducing equivalents. In addition to its capability for MMb reduction, the NADH produced in the reaction is not used by the reversible product of the enzymatic reaction because GOT (naturally present in muscle) in the presence of glutamate utilizes oxaloacetate and converts it to α-ketoglutarate. Moreover, NADH produced via the malate–MDH reaction pathway was active and stable at a pH typical of meat (5.5–5.8).

Our data suggest a direct relationship between change in pH, NADH regeneration, and MMb reduction via the malate–MDH pathway (**Figure 1B**). In the present study, when hydroxymalonic acid (an MDH inhibitor) was used as substrate, MMb reduction rate was substantially slower, presumably because of the specificity of MDH for L-malate.

Many enzymes, both glycolytic and mitochondrial, remain active in post-mortem meat (*5*) and may have a crucial role in enzymatic reduction of MMb, in which the post-mortem cytoplasmic and glycolytic pool of NADH is used by the NADH–cytochrome b_5 reductase system in muscle (*22*). Furthermore, Saleh and Watts (*23*) tested the ability of several glycolytic and Krebs cycle intermediates (including malate) to increase MMb reduction in ground beef and concluded that addition of appropriate substrates can increase the reducing activity of most meat samples. However, under physiological conditions, the ratio of cytosolic-free NAD^+ to NADH is approximately 700 to 1 (*24*), whereas the ratio of mitochondrial NAD^+ to NADH is 7–8 to 1 (*25*, *26*). Williamson et al. (*25*) reported that the mitochondrial NADH pool represents a significant portion of the total NADH pool. Additionally, the NADH pool produced from glycolytic flux is mostly transferred to mitochondria through the malate–aspartate shuttle system (*27*). Therefore, our primary interest was to investigate the ability of MDH, an oxidoreductase enzyme prevalent in both cytosol and mitochondria, to regenerate NADH and concomitantly reduce MMb.

Reduction of Metmyoglobin by Skeletal Muscle Extracts. The purpose of this investigation (experiment 2) was to provide evidence for potential malate interactions with myoglobin redox chemistry in post-mortem bovine muscle. Furthermore, this study was performed to investigate the hypothesis that *in vitro* MMb

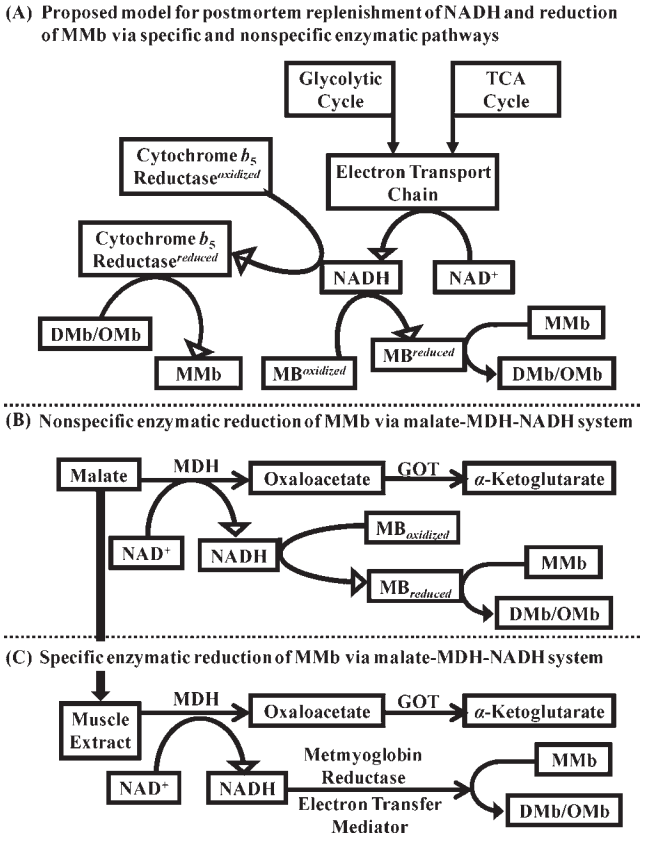

Figure 6. Schematics of proposed pathways of (**A**) using enzymes of glycolytic and tricarboxylic acid cycle for MRA in meat, (**B**) nonspecific enzymatic reduction, and (**C**) specific enzymatic reduction of MMb via a malate−MDH−NADH system with generation of NADH.

reduction is related to malate's ability to replenish the post-mortem pool of NADH via MDH activity.

The kinetic data of malate interactions with MMb reductase of muscle extracts from the current study provide direct evidence that extracts from beef skeletal muscles contains enzymes that (1) can use malate as substrate, (2) help replenish the post-mortem pool of NADH in extracts (**Figure 4**), and (3) can couple the NADH with MMb reduction through interactions and electron transfer mediation with MMb reductase. The kinetic data (**Figure 5A−C**) also suggest that MDH present in muscle extracts require NAD^+ and malate for its enzymatic function and that MMb-reducing enzymes present in muscle extracts require NADH for subsequent MMb reduction. Activity of MDH in extracts from three different bovine muscles supports the original concept proposed by Watts et al. (*6*) that post-mortem muscle can replenish NADH by reducing NAD^+ and that a NADH-dependent reducing system, either specific enzymatic or nonspecific enzymatic, can reduce MMb (**Figure 6A−C**). Our data agree with numerous reports indicating that muscles differ in their color stability due to a complex interaction between their inherent muscle physiology and biochemistry, the changes that occur in each muscle during its conversion from pre- to postrigor tissue, and the processing protocol of the meat (*2−4*, *11*, *12*, *14*, *28−30*).

Because enzymatic reduction of MMb in meat occurs primarily through a NADH-dependent MMb reductase (*28*), a mechanism that supports the hypothesis that interaction of a malate−MDH system with MMb reductase extracted from bovine skeletal muscle *in vitro* will provide greater insight into understanding the effects of malate on meat color. It is well established that MMb reduction occurs through both specific enzymatic and nonspecific enzymatic reducing systems and that NADH is the preferred reducing substrate for both pathways (*2*). Giddings (*13*) reviewed enzymatic reduction of MMb in meat, and numerous subsequent investigations of MMb reduction in meat systems have provided evidence for the phenomenon but have not explained the mechanism by which it occurs (*29−32*). Brown and Snyder (*33*) presented evidence for nonspecific enzymatic reduction of MMb. They reported that NADH and NADPH acted as an active reductants and their reducing ability was enhanced by addition of flavins and methylene blue. Several MMb reductases that require NADH and an appropriate mediator to facilitate conversion of ferric myoglobin to its ferrous form have been described (*34−36*).

In the present study, importance of the malate−aspartate shuttle was investigated for the first time in reference to MMb reduction and the capability of post-mortem muscle extracts from different bovine muscles to reduce MMb. The results demonstrate the crucial role of the malate−MDH system in post-mortem replenishment of NADH and subsequent mediated electron transfer to and reduction of MMb. Our data clearly show a predominant role for malate, a key Krebs cycle intermediate, in MMb reduction and an apparent role in stabilizing myoglobin redox chemistry in post-mortem skeletal muscles. Furthermore, addition of malate to muscle extracts provides an excellent model for studying MMb reduction and may provide a means of improving meat color by using components of meats with low commercial value.

ABBREVIATIONS USED

LD, M. longissimus dorsi; PM, M. psoas major; ST, M. semitendinosus; DMb, deoxymyoglobin; OMb, oxymyoglobin; MMb, metmyoglobin; EDTA, ethylenediaminetetraacetic acid; FMN, flavin mononucleotide; GOT, glutamate-oxaloacetate transaminase; HMA, hydroxymalonic acid; MB, methylene blue; MDH, malate dehydrogenase; MRA, metmyoglobin-reducing activity; NAD^+, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide.

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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