JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 7021–7029 7021
DOI:10.1021/jf100714g

Myoglobin Redox Form Stabilization by Compartmentalized Lactate and Malate Dehydrogenases

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

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

The purpose of this study was to assess the ability of mitochondrial and cytoplasmic malate dehydrogenase present in postrigor bovine skeletal muscle to use malate as a substrate for reduced nicotinamide adenine dinucleotide (NADH) regeneration and metmyoglobin (MMb) reduction via the malate−NAD^+−MMb system. Furthermore, addition of lactate to beef mitochondrial and cytoplasmic isolates was evaluated to determine whether interactions between malate and lactate increased MMb reduction. Addition of malate to isolated beef mitochondrial and cytoplasmic isolates at pH 7.2 increased ($p < 0.05$) MMb reduction. MMb reduction resulting from addition of malate and lactate was equal to or greater than MMb reduction resulting from malate alone. This suggests that a combination of mitochondrial (malate) and cytoplasmic (lactate) factors can be used to regenerate the post-mortem pool of NADH, resulting in metmyoglobin reduction and meat color stabilization.

KEYWORDS: Metmyoglobin; malate; MDH; lactate; MRA; meat color; NADH

INTRODUCTION

Fresh meat color is determined by the redox status and presence of relative proportions of myoglobin redox forms: deoxymyoglobin (DMb), oxymyoglobin, and metmyoglobin (MMb). Central packaging of case-ready meats uses numerous ingredients that add value and extend product shelf life. Myoglobin, the protein responsible for red color, interacts with these ingredients through complex biochemical reactions, affecting myoglobin redox chemistry and meat color stability. Pathways by which these ingredients interact with myoglobin redox chemistry are unclear, but understanding the biochemical mechanisms that stabilize myoglobin redox form can offer novel insights into meat color chemistry, enabling development of new strategies to improve beef color stability.

Mitochondria are important subcellular organelles involved in energy metabolism. Several researchers (*1*−*3*) have reported that myoglobin redox stability and mitochondrial activity in postmortem skeletal muscles are interrelated. Mitochondria have the potential to affect myoglobin redox stability and meat color by mediating MMb reduction via tricarboxylic acid (TCA) metabolites by the transfer of reducing equivalents between extra- and intramitochondrial membranes (*3*). Andrews et al. (*4*) and Bodwell et al. (*5*) pointed out that enzymes involved in glycolysis and the TCA cycle remain active in post-mortem muscle and, therefore, could be possible sources of reducing equivalents. Other evidence (*2*, *3*, *6*−*8*) suggests that metabolic activity of mitochondria in post-mortem muscles may have a more direct role in myoglobin redox form stability.

Tang et al. (*6*) reported that mitochondria isolated from bovine cardiac muscle 60 days post-mortem had decreased consumed oxygen and respiratory control ratios compared with mitochondria isolated at 96 h post-mortem. Oxygen consumption at 60 days was only 10−15% of that at 2 h post-mortem when measured at pH 5.6 and 7.2. Post-mortem metabolic potential of mitochondria also decreased as pH decreased. State III and IV oxygen consumption decreased as pH decreased from 7.2 to 6.4 to 5.6, with the most substantial decline from pH 6.4 to pH 5.6 (*6*). Arihara et al. (*9*) suggested that mitochondria in post-mortem skeletal muscles maintain metabolic activity for a long period of time and that enzymes located in mitochondria may reduce MMb. Tang et al. (*1*) suggested that enzymes responsible for MMb reduction are located within the muscle's mitochondria and that the mitochondrial pool of NADH should provide for metmyoglobin-reducing activity (MRA). However, it is unclear how such enzymes within muscle mitochondria may relate to MRA.

Several researchers (*10*−*12*) indicated that meat discoloration was more related to muscle mitochondrial enzyme activity than mitochondrial content. Tang et al. (*6*) reported that a decrease in oxygen partial pressure resulted in decreased mitochondrial respiration and more MMb formation. Muscles with high mitochondrial content 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 facilitate MMb reduction by supplementing meat tissue with a post-mortem pool of reduced cofactors generated by reversal of the electron transport chain. Tang et al. (*1*, *6*) concluded that addition of succinate to a bovine mitochondria−myoglobin system resulted in both oxygen consumption and MMb reduction. Bodwell et al. (*5*) suggested that availability of substrates to TCA enzymes (dehydrogenases) that help to regenerate reducing equivalents in post-mortem skeletal muscles

*Corresponding author. Tel: 785-532-1232. Fax: 785-532-7059. E-mail: hhunt@ksu.edu.

© 2010 American Chemical Society
Published on Web 05/14/2010

could be the limiting factor. Collectively, these observations suggest that mitochondria play an important role in meat color stability.

The effects of compartmentalization of enzymes between mitochondria and cell sap [such as malate dehydrogenase (MDH)] have not been reported relative to their ability to generate reducing equivalents and influence myoglobin redox forms in beef muscles differing in inherent color stability. Klingenberg and Bucher (*14*) reported that a bimodal distribution of enzymes between mitochondria and cell sap is characteristic of several enzymes including MDH. More specifically, NADH resulting from MDH activity between mitochondria and cell sap might play an important role in meat color stability.

MMb may be reduced nonenzymatically (*15*−*18*) or enzymatically (*16*, *17*, *19*−*21*) by enzymes located within muscle mitochondria (*9*, *22*) such as cytochome *c* oxidase (CcOx). Reduction of MMb occurs in the presence of mitochondria, lactate, and succinate *in vitro* (*1*, *3*, *6*). Thus, mitochondria as a source of NADH, a key component of MRA, should provide for MRA (*7*). It is not known, however, how CcOx activity within muscles may relate to MRA.

Moreover, a metabolite-induced regeneration of NADH by compartmentalized enzymes such as MDH and lactate dehydrogenase (LDH) that may mediate MMb reduction and influence myoglobin redox stability has not been reported. More specifically, NADH production resulting from MDH and/or LDH activity between mitochondria and cell cytoplasm might play an important role in meat color stability. Therefore, we hypothesized that NADH produced from MDH and/or LDH has the potential to improve myoglobin redox stability. Objectives of this study were to assess (1) the functional potential of compartmentalized MDH and LDH from mitochondria and cell cytoplasm for MMb reduction and (2) relationships among color stability mediated by compartmentalized MDH and LDH, MRA, and CcOx activity for three beef muscles differing in color stability.

MATERIALS AND METHODS

Chemicals. Equine skeletal muscle myoglobin, magnesium chloride ($MgCl_2$), bovine serum albumin (BSA), sucrose, tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), ethylenediaminetetraacetic acid (EDTA), potassium phosphate monobasic (KH_2PO_4), sodium malate, potassium chloride (KCl), and Nagarse protease (10.5 units/mg) were obtained from Sigma Chemical Co. (St. Louis, MO); sodium succinate was purchased from Fisher Scientific (Fair Lawn, NJ). All chemicals were of reagent grade.

Raw Materials. Five beef carcass sides representing different animals (USDA Select, A level maturity, normal color, and absent of quality defects) were selected randomly 2 days post-mortem at a commercial abattoir. *Longissimus lumborum* (LL), *Psoas major* (PM), and *Semitendinosus* (ST) muscles were obtained from each carcass side and stored in vacuum for either 10 or 20 days post-mortem at 2 ± 1 °C. Because fiber types vary in these muscles (*23*), tissue samples were taken from the central portion of the LL, the whole cross-sectional portion of the PM, and the outer portion of the ST (the small deep red portion of the ST was excluded).

pH. Samples visually devoid of intermuscular fat and connective tissue were frozen in liquid nitrogen and blended in a Waring table-top blender (Dynamics Corp. of America, New Hartford, CT). To determine pH, 10 g of pulverized sample was combined with 100 mL of deionized water and mixed for 30 s, and pH values were obtained by using an Accumet combination glass electrode connected to an Accumet 50 pH meter (Fisher Scientific, Fairlawn, NJ).

Proximate Analysis. Ten grams of sample, visually devoid of intermuscular fat and connective tissue, was frozen in liquid nitrogen and pulverized for proximate analysis. Samples were analyzed (n = 5) for protein [LECO Combustion Analysis (AOAC Official Method 990.03) (*24*)] and moisture and fat [CEM SMART and SMART Trac systems (AOAC PVM 1:2003) (*25*)].

Mitochondria Isolation. Mitochondria were isolated according to the methods of Bhattacharya et al. (*24*) and a slightly modified method of Frezza et al. (*27*). All mitochondrial isolation procedures were performed at 0−4 °C. Ten grams of muscle, devoid of fat and connective tissue, was homogenized in 50 mL of isolation buffer 1 (46 mM KCl, 100 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM EDTA, 0.5% BSA, pH 7.4) with a homogenizer (Kinematica Polytron benchtop Model PT 3100; Brinkmann, Lucerne, Switzerland). After homogenization, 25 mg of proteinase K was mixed, and the sample was incubated for 5 min at 0 °C before 100 mL of isolation buffer 1 was added. Further homogenization at 0 °C was accomplished with a Kontes Duall grinder (Vineland, NJ) and then with a Wheaton Potter-Elvehjem grinder (Millville, NJ). All Teflon grinding pestles rotated at 1400 rpm. Each sample was centrifuged at 600*g* at 0 °C for 10 min (Sorvall RC-5B, Newtown, CT) and filtered through two layers of cheesecloth. The supernatant was centrifuged at 14000*g* at 0 °C for 10 min, and the resulting pellet was vortexed with 5 mL of isolation buffer 2 (0.1 M KCl, 50 mM Tris-HCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.5% BSA, pH 7.4). Samples were centrifuged again at 7000*g* at 0 °C for 10 min, and the resulting pellet was vortexed with isolation buffer 2. Final centrifugation was accomplished at 3500*g* at 0 °C for 10 min, and the isolated mitochondria pellet was vortexed with 0.25 M sucrose. Mitochondrial and cytoplasmic protein content was determined with a bicinchoninic acid protein assay kit from Sigma Chemical Co. (St. Louis, MO).

MRA Assay with Isolated Mitochondrial and Cytoplasmic Protein. Activity of enzymes was assessed spectrophotometrically (UV-2010; Hitachi Instrument, Inc., San Jose, CA) at 30 °C and pH 7.0. The LDH and MDH enzyme activity was assessed in the cytoplasmic and mitochondrial fractions. Each assay contained various amounts of mitochondrial and cytoplasmic proteins added to obtain a rate of 0.1 absorbance unit min^{-1}. Activities were reported as nanomoles of MMb reduced per milligram per minute. MDH and LDH activities were measured by following the rate of NADH oxidation at 340 nm (millimolar extinction coefficient = 6.23). The mitochondrial fraction was diluted in 10 mM $KHPO_4$ and subjected to three freeze−thaw cycles. Lactate and malate were used as substrates to assess maximal rates of MMb reduction. Mitochondrial samples were diluted in 10 mM $KHPO_4$ and subjected to sonication (Branson Sonifier 250) at 40% full power.

Cytochrome *c* Oxidase Activity. CcOx activity was determined with a colorimetric assay kit (Sigma Chemical Co., St. Louis, MO). Samples for CcOx were removed on days 10 and 20 from each muscle type, frozen instantly in liquid nitrogen, vacuum packaged, and stored at −80 °C until analysis. Mitochondria were isolated from skeletal muscle samples according to the procedure mentioned elsewhere. Determination of cytochrome *c* oxidase activity was based on a colorimetric assay that quantifies oxidation of ferrocytochrome *c* to ferricytochrome *c* via CcOx, a reaction that results in a decrease in absorbance at 550 nm (Sigma cytochrome *c* oxidase technical bulletin). The decrease in absorbance at 550 nm was monitored with a spectrophotometer (UV-2010; Hitachi Instrument, Inc., San Jose, CA). The spectrophotometer was calibrated to zero with assay buffer (10 mM Tris-HCl and 120 mM KCl, pH 7.0, 25 °C).

In a cuvette, 0.95 mL of assay buffer was combined with 50 μL of enzyme buffer (10 mM Tris-HCl and 250 mM sucrose, pH 7.0, 4 °C) and 50 μL of isolated mitochondria. The reaction was initiated by adding 50 μL of ferrocytochrome *c* (reduced with 0.1 M dithiothreitol), and the decrease in absorbance at 550 nm was measured every second for 1 min by using a kinetics program. Activity (units per milliliter) was calculated by using the equation [(ΔAbs_{550}/min for the sample − ΔAbs_{550}/min for the blank) × dilution factor (6) × total reaction volume (1.1 mL)]/[mitochondria isolate volume (0.05 mL) × the difference in extinction coefficients between ferro- and ferricytochrome *c* at 550 nm (21.84)]. One unit will oxidize 1 μM reduced cytochrome *c*/min at pH 7.0 and 30 °C.

Metmyoglobin-Reducing Activity. MRA was determined by using a 2.54 cm cube from each steak or region within a steak on days 0, 4, and 7 according to a procedure described by Sammel et al. (*26*). Samples were submerged for 20 min in a 0.3% solution of sodium nitrite to facilitate nitric oxide metmyoglobin (MMb) formation, removed, blotted dry, and vacuum packaged at 62.2 cmHg vacuum (Multyivac C500, Multivac Inc., Kansas City, MO). On the light-exposed display surface, samples were scanned twice with a HunterLab MiniScan XE Plus spectrophotometer (D/8-S, 14.3 mm diameter aperture; Hunter Associates Laboratory, Inc., Reston, VA) to obtain 400−700 nm reflectance data. Samples were

Table 1. LS Means ($n = 5$) for pH, Moisture, Crude Protein, and Fat of Three Different Beef Muscles

	muscles[a]			
trait	LL	PM	ST	SE[b]
pH	5.8[x]	5.6[x]	5.7[x]	0.04
moisture, %	71.3[x]	71.2[x]	74.1[y]	0.12−0.47
crude protein, %	23.6[z]	21.9[x]	22.4[y]	
fat, %	4.2[y]	1.4[x]	1.5[x]	
ash, %	5.1[x]	5.5[x]	2.0[y]	

[a] LL = *Longissimus lumborum*; PM = *Psoas major*; ST = *Semitendinosus*. [x−z] Means in a row with a different letter differ ($p < 0.05$). [b] Standard error.

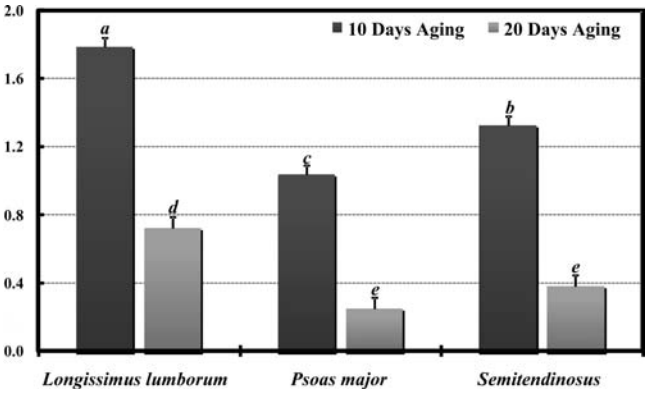

Figure 1. Cytochrome *c* oxidase (CcOx) activity of three bovine muscles (*Longissimus lumborum*, *Psoas major*, *Semitendinosus*) aged for 10 and 20 days. Metmyoglobin reducing activity (MRA), or % MMb reduced, was calculated as ((Δ % surface MMb)/(preincubation % surface MMb)) × 100. a, b, c, d, e = means across muscles by aging time with a different letter differ ($p < 0.05$).

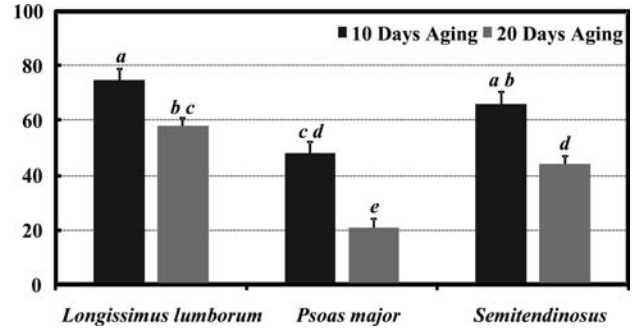

Figure 2. Metmyoglobin-reducing activity (MRA) of three bovine muscles (*Longissimus lumborum*, *Psoas major*, *Semitendinosus*) aged for 10 and 20 days. MRA, or % MMb reduced, was calculated as ((Δ % surface MMb)/(preincubation % surface MMb)) × 100. a, b, c, d, e = means across muscles by aging time with a different letter differ ($p < 0.05$).

incubated at 30 °C for 2 h (Thelco model 4; Precision Scientific, Chicago, IL) to induce nitric oxide MMb reduction to DMb. Upon removal from the incubator, samples were rescanned twice immediately to determine the percentage of remaining surface MMb using K/S ratios and equations from AMSA (*29*). The following equation was used to calculate MRA: ((Δ % surface MMb)/(preincubation % surface MMb)) × 100.

Statistical Analysis. The experimental design for determining muscle effects on MDH, LDH, and CcOx activity was a randomized complete block. Type 3 tests of fixed effects for muscle were performed by using the MIXED procedure of SAS (SAS, 2007, Release 9.1.3; SAS Institute Inc., Cary, NC), and beef carcass sides ($n = 5$ animals) were considered random blocking effects. The experimental designs for determining muscle effects (whole plot fixed effect) on MRA were split plots. The whole plot consisted

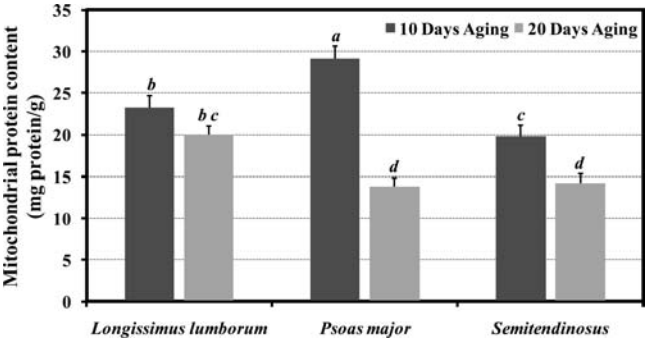

Figure 3. Mitochondrial protein content for three bovine muscles (*Longissimus lumborum*, *Psoas major*, *Semitendinosus*) aged for 10 and 20 days. a, b, c, d = means across muscles by aging time with a different letter differ ($p < 0.05$).

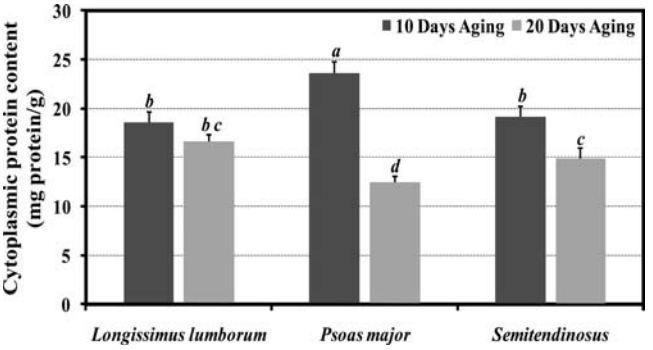

Figure 4. Cytoplasmic protein content for three bovine muscles (*Longissimus lumborum*, *Psoas major*, *Semitendinosus*) aged for 10 and 20 days. a, b, c, d = means across muscles by aging time with a different letter differ ($p < 0.05$).

of a randomized complete block in which beef carcass sides ($n = 5$ animals) served as blocks. Time of chemical analysis (subplot treatment) was assigned to one of the three steaks (subplot unit) from each muscle (15 total units; five animals × three muscle types). Type 3 tests of fixed effects for muscle, muscle types, and their interaction were performed by using the MIXED procedure of SAS. Beef carcass sides (animals) were considered random effects for the randomized-complete-block portion of the whole plot, and muscle × animal was used to determine whole-plot error. Least squares means for *F*-tests ($p < 0.05$) were separated using the diff option (least significant differences) and were considered significant at $p < 0.05$.

RESULTS

pH and Proximate Analysis. The PM had the lowest pH but all muscles were between pH 5.5 and pH 5.7 (**Table 1**).

Cytochrome *c* Oxidase Activity. CcOx activity was greatest ($p < 0.05$) for the LL, intermediate for the ST, and lowest for PM for aging day 10 (**Figure 1**). CcOx activity for the ST and PM was similar ($p > 0.05$) on day 20 and was less ($p < 0.05$) than that of the LL. Aging from day 10 to day 20 decreased ($p < 0.05$) CcOx activity for all muscle types.

Metmyoglobin-Reducing Activity. MRA decreased ($p < 0.05$) from 10 to 20 days of aging for all muscles (**Figure 2**). The decrease in MRA was least for the LD, intermediate for the ST, and greatest for the PM for both aging times. The MRA for the ST aged 10 days was not different ($p > 0.05$) from that of the LD aged 10 and 20 days. The MRA of the PM aged 10 days was not different ($p > 0.05$) from that of the ST aged 20 days. The PM had the lowest MRA for both aging times.

Mitochondrial Protein Content. Mitochondrial concentration (milligrams of mitochondrial protein per gram of muscle) for each muscle decreased from 10 to 20 days of aging, but the decline for the LD was not significant (**Figure 3**). Mitochondrial concentration

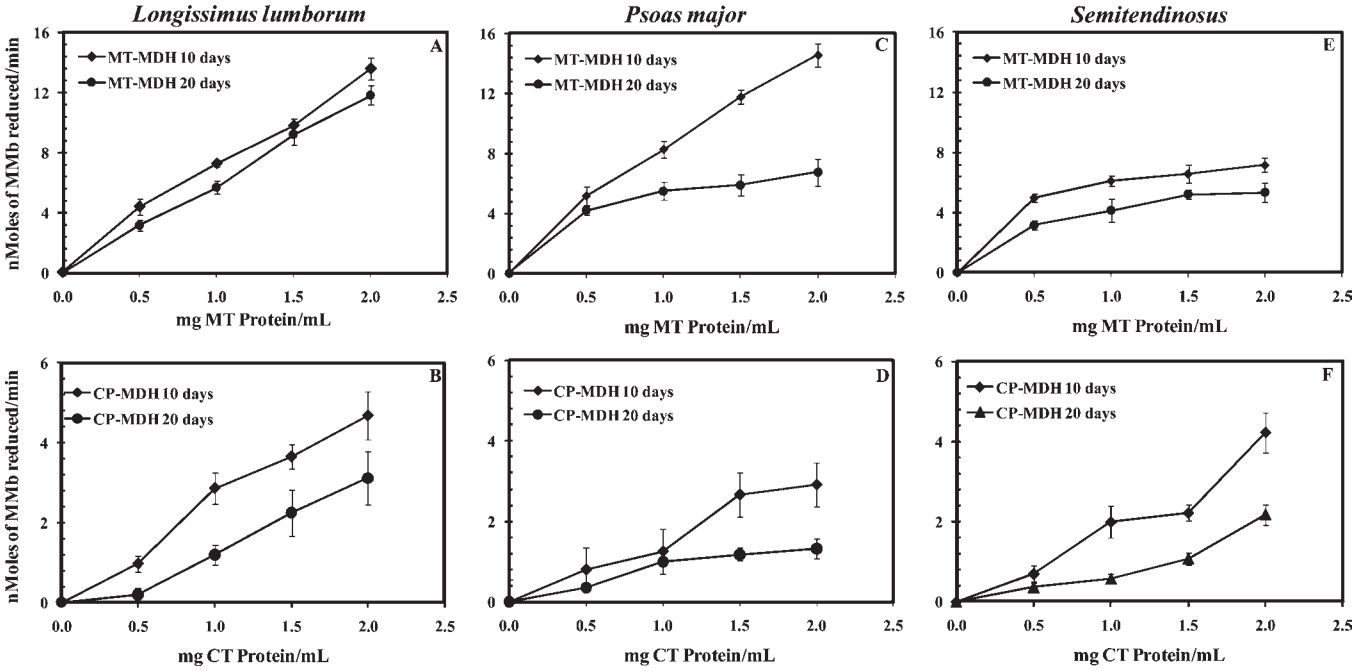

Figure 5. Kinetics of metmyoglobin reduction using mitochondrial and cytoplasmic malate dehydrogenase (m-MDH and c-MDH, respectively; pH 7.0). Concentration effects of the amount of individual muscle mitochondrial and cytoplasmic isolates obtained from three beef muscles (*Longissimus lumborum*, *Psoas major*, *Semitendinosus*) on the MRA of mitochondrial and cytoplasmic isolates.

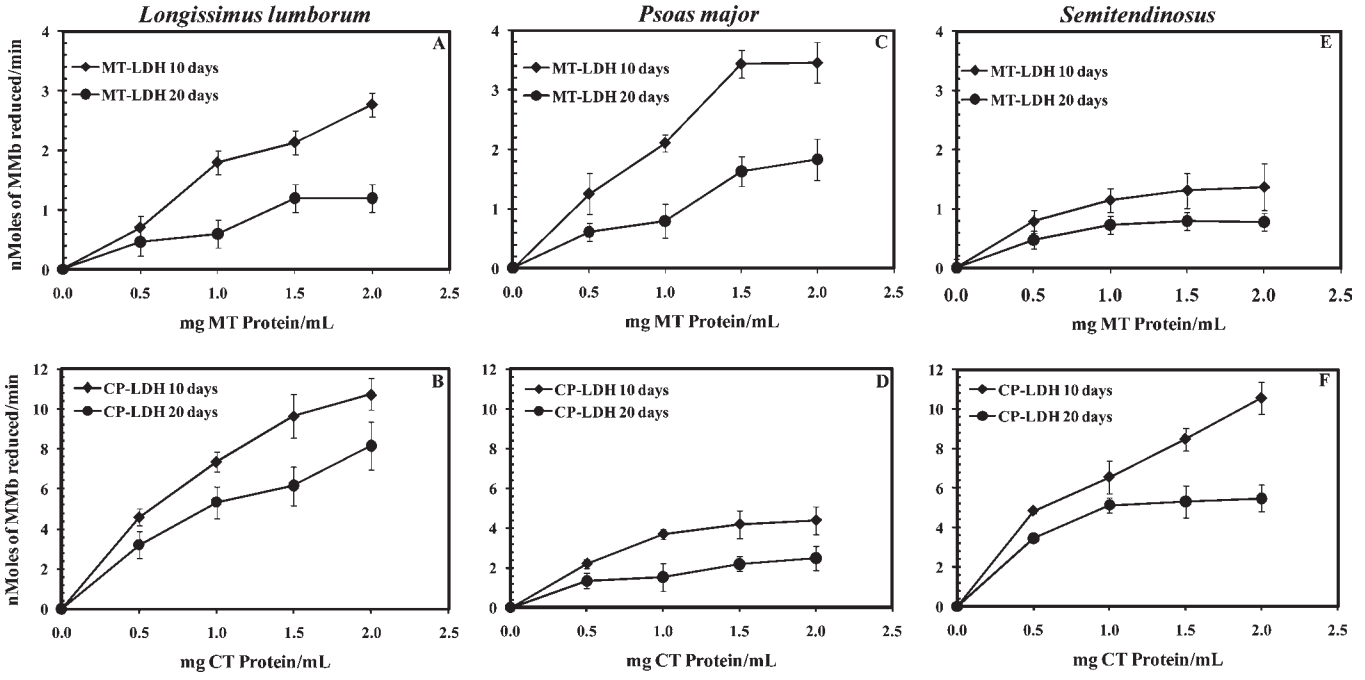

Figure 6. Kinetics of metmyoglobin reduction using mitochondrial and cytoplasmic lactate dehydrogenase (m-LDH and c-LDH, respectively; pH 7.0). Concentration effects of the amount of individual muscle mitochondrial and cytoplasmic isolates obtained from three beef muscles (*Longissimus lumborum*, *Psoas major*, *Semitendinosus*) on the MRA of mitochondrial and cytoplasmic isolates.

was greatest for the PM at day 10, intermediate for the LL at 10 and 20 days and the ST at 10 days, and lowest for the PM and ST at 20 days. In general, mitochondrial concentration for both aging times tended to be lowest for the ST, intermediate for the LL, and highest for the PM.

Cytoplasmic Protein Concentration. Cytoplasmic protein concentration (**Figure 4**) for the PM at 10 days of aging was greater ($p < 0.05$) than that of the LL and ST at 10 and 20 days. Cytoplasmic protein concentration was lowest for PM at 20 days, intermediate for ST and LL, and highest ($p < 0.05$) for PM at 10 days. The PM had the greatest decrease ($p < 0.05$) in cytoplasmic protein due to aging.

Reduction of Metmyoglobin by Mitochondrial and Cytoplasmic Malate Dehydrogenase. Kinetic data (**Figure 5**) showed differences in MMb reduction between beef muscles aged for 10 and 20 days. Increasing concentrations of mitochondrial protein (0.5, 1.0, 1.5, and 2.0 mg g^{-1}) isolated from LL and PM muscles aged for 10 days resulted in greater ($p < 0.05$) MMb reduction via

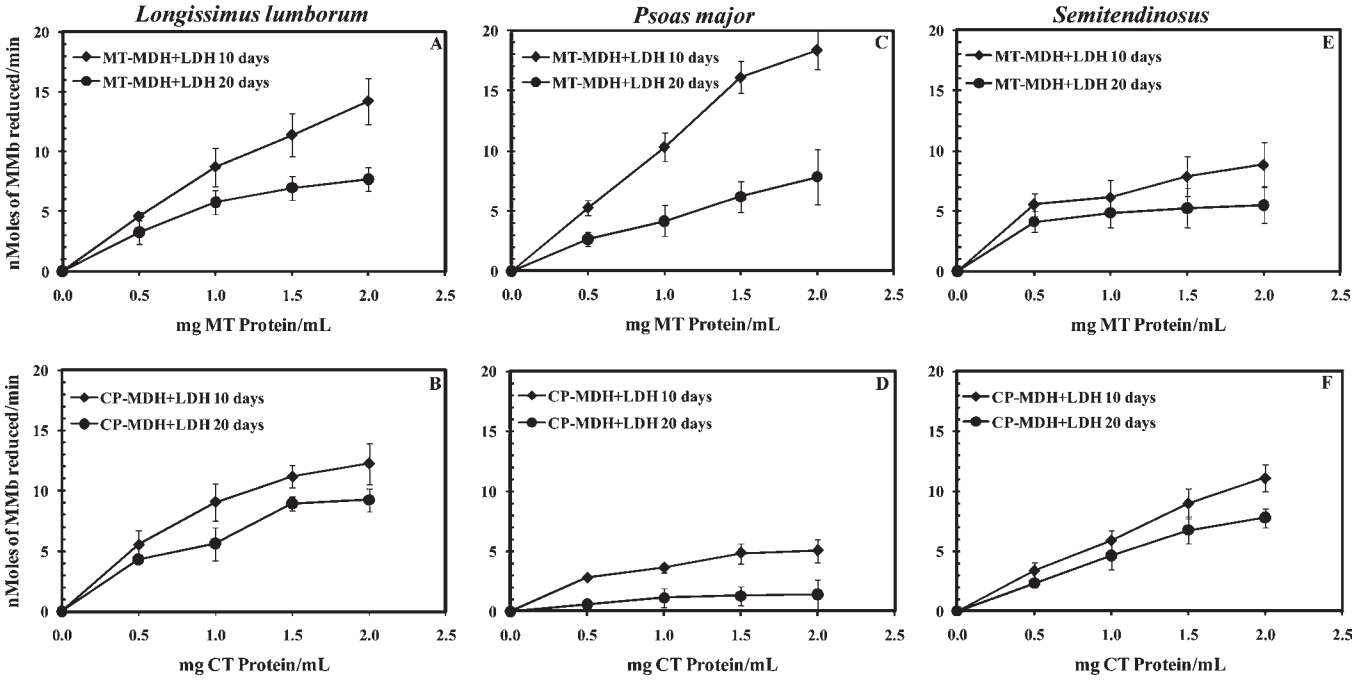

Figure 7. Kinetics of metmyoglobin reduction using mitochondrial and cytoplasmic lactate and malate dehydrogenases (m-LDH, c-LDH, m-MDH, and c-MDH, respectively; pH 7.0). Concentration effects of the amount of individual muscle mitochondrial and cytoplasmic isolates obtained from three beef muscles (*Longissimus lumborum*, *Psoas major*, *Semitendinosus*) on the MRA of mitochondrial and cytoplasmic isolates.

malate−MDH pathways than that from LL and PM muscles aged for 20 days. Reduction of MMb by mitochondrial MDH (m-MDH) was greater ($p < 0.05$) than that by cytoplasmic MDH (c-MDH) for all three beef muscles. A similar trend was observed for MMb reduction by m-MDH and c-MDH isolates from ST for both aging periods. Kinetic data for the m-MDH-assisted MMb reduction indicated that PM exhibited increased MMb reduction compared with LL and ST muscles aged 10 days. However, there were no differences in m-MDH-assisted MMb reduction for all three beef muscles aged 20 days. In general, MMb reductions by m-MDH and c-MDH were different ($p < 0.05$), and reduction of MMb by m-MDH was greater than that by c-MDH for all three beef muscles.

Reduction of Metmyoglobin by Mitochondrial and Cytoplasmic Lactate Dehydrogenase. Kinetic data showed (**Figure 6**) that cytoplasmic LDH (c-LDH) exhibited greater MMb reduction rates than mitochondrial LDH (m-LDH). Increased concentrations of mitochondrial protein (0.5, 1.0, 1.5, and 2.0 mg/g) isolated from the PM showed greater ($p < 0.05$) MMb reduction than that from the LL and ST muscles aged for 10 and 20 days. Increasing concentrations of cytoplasmic protein (0.5, 1.0, 1.5, and 2.0 mg/g) isolated from LL muscle aged for 10 and 20 days showed differences ($p < 0.05$) in MMb reduction. Reduction of MMb by m-LDH was much lower ($p < 0.05$) than that by c-LDH for all three beef muscles. MMb reduction kinetics using m-LDH from PM muscles aged for 10 days had greater ($p < 0.05$) MMb reduction than m-LDH from PM muscle aged for 20 days. Also, c-LDH activity for MMb reduction by PM was lower than that for ST and LL. Also, c-LDH activity and MMb reduction were greater for the LL than for the PM and ST. Beef muscles aged from 10 to 20 days tended to have lower c-LDH and m-LDH activities for facilitating MMb reduction.

Reduction of Metmyoglobin by Mitochondrial and Cytoplasmic Malate and Lactate Dehydrogenases. Addition of mixtures of malate and lactate to the beef skeletal muscle mitochondrial and cytoplasmic isolates enhanced MMb reduction compared with addition of lactate or malate alone. Of the three beef muscles, addition of malate and lactate to mitochondrial isolates from the PM exhibited greater MMb reduction than addition to mitochondrial isolates from the LL and ST aged for 10 days (**Figure 7**). In contrast, cytoplasmic protein isolated from the PM had minimal MMb reduction compared with that isolated from the LL and ST. Mitochondrial and cytoplasmic protein isolated from the LL and ST aged for 10 and 20 days showed a similar trend for MMb reduction. Kinetic data clearly showed that the rate of MMb reduction was concentration dependent and that increasing concentrations of cytoplasmic and mitochondrial protein increased MMb reduction in muscles aged for 10 and 20 days compared with the control.

DISCUSSION

Reduction of Metmyoglobin by Compartmentalized Malate Dehydrogenase. Primary objectives of this experiment were to (1) investigate the ability of compartmentalized TCA enzyme MDH from post-mortem beef skeletal muscle to use substrate (sodium malate) supplementation to regenerate reducing equivalents and (2) evaluate the ability of compartmentalized MDH to mediate MMb reduction and influence myoglobin redox stability via malate−MDH pathways. Our investigations provide substantial evidence indicating that addition of malate to mitochondrial and cytoplasmic isolates leads to MMb reduction via regeneration of NADH and malate−MDH pathways.

Several enzymes in the TCA cycle are capable of reducing NAD^+. However, the position of m-MDH in the TCA cycle combined with the results of this experiment (which show considerable specific activity of m-MDH for NAD^+-linked MDH in beef skeletal muscle mitochondria capable of reducing MMb) represents a potential mechanism by which substrates of TCA enzymes could be used as a source of NADH post-mortem and plays a direct role in myoglobin redox form stability. Although this investigation did not comprehensively evaluate all compartmentalized enzymes, it appears that the compartmentalized MDH differ not only in their relative ability to regenerate a post-mortem pool of NADH but also in their ability to reduce MMb at different rates in different muscles.

Our results also provide evidence that enzymatic compositions, specifically of c- and m-MDH, of the three beef skeletal muscles differ considerably. Addition of malate to the mitochondrial isolate from PM resulted in the greatest MMb reduction rates, whereas mitochondrial isolates from LL and ST did not show significant differences in their ability to reduce MMb.

Although the morphological makeup of mitochondria in muscles composed of red or white fibers may not be different (*2*, *6*), it is the concentration and functional integrity of the mitochondria post-mortem that may lead to greater color stability in muscles (*13*, *30*). Of the muscles used in this study, ST has the greatest amount of α-white fibers, LL has slightly more α-red and α-white, and PM is mostly β-red and α-red fibers (*31*). Muscles high in β-red and α-red fibers, such as PM, have greater mitochondrial and heme protein concentrations, and they bloom (oxygenate) more slowly than other muscles because of increased competition for available oxygen by mitochondria and other organelles. Muscles composed mainly of white fibers have a lower consumption of oxygen (*32*). Therefore, muscles with more glycolytic fibers, such as LL and ST, bloom brightly at the initiation of display because oxygen binds to myoglobin without mitochondrial competition. Consequently, the LL and ST should have greater color stability, and the PM should be more color labile.

Bodwell et al. (*5*) and Watts et al. (*19*) suggested that availability of substrates to TCA enzymes (dehydrogenases) that help to regenerate reducing equivalents in post-mortem skeletal muscles could be the limiting factor in meat color stability. Tang et al. (*1*, *6*) reported that addition of succinate to bovine mitochondria resulted in greater oxygen consumption and MMb reduction. Lanari and Cassens (*12*) reported that mitochondrial and submitochondrial particles facilitated myoglobin redox stability and suggested that mitochondria content and post-mortem mitochondrial enzyme activity were more directly related to meat discoloration. Renerre (*10*) found that beef skeletal muscles with high mitochondrial content were associated with high oxidative metabolism and low color stability.

Malic acid, a natural constituent of many fruits and vegetables and a metabolite of the TCA cycle, is involved in production of energy in the body under both aerobic and anaerobic conditions through the malate−aspartate redox shuttle (**Figure 8**) via the electron transport chain. As a result of malic acid's role in the malate−aspartate redox shuttle and the TCA cycle, NADH is produced. The malate−aspartate shuttle is the principal means for removing reducing equivalents from the cytoplasm to the mitochondria (*33*). Intramitochondrially, malate is oxidized to oxaloacetate by the TCA cycle, regenerating a mitochondrial pool of reducing equivalents that ultimately participates in the electron transport chain and may reduce MMb near the mitochondrial outer membrane as proposed in the model shown in **Figure 8**.

According to the model of McElroy, Wong, and Williams (*34*), intramitochondrial oxaloacetate formed in this way is transaminated and reduced to malate. This process allows the transfer of the electrons from extramitochondrial NADH to malate, which reenters the mitochondria. This model further supports the finding that the energy-rich mitochondria should then export malate nonenergetically (as a source of cytoplasmic NADH) and import malate in the energy-poor state.

Although the role of m-MDH in regeneration of mitochondrial pool of NADH via TCA pathways is well documented (*35*), the role of c-MDH in generation of reducing equivalents is less clear. The usefulness of having two localized isoenzymes may be a very critical factor when, as in postrigor skeletal muscle, NADH is the most limiting factor for myoglobin redox stability. Moreover, NADH produced by the activity of lactate dehydrogenase cannot

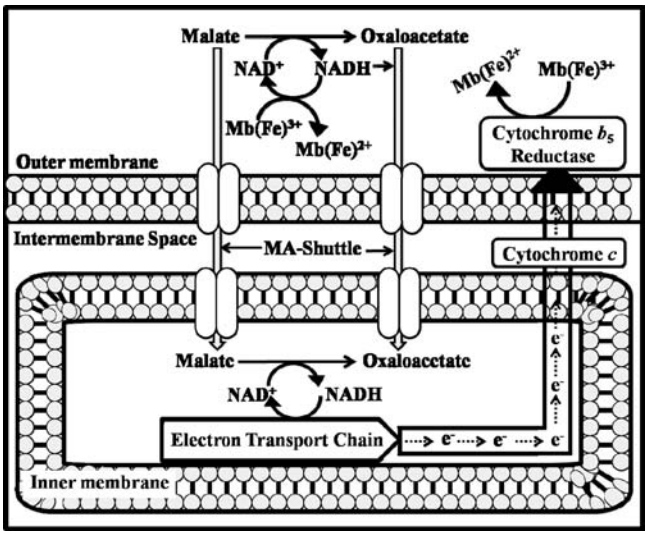

Figure 8. Proposed model and biochemical pathways for enzymatic and nonenzymatic reduction of metmyoglobin and depiction of the NADH regeneration via cytoplasmic and mitochondrial malate−MDH activity. Entry of malate into the mitochondrial matrix is facilitated by the malate−aspartate shuttle (MA-Shuttle). Mitochondrial MDH (m-MDH) oxidize malate to oxaloacetate, regenerating the post-mortem pool of NADH and further oxidzing NADH via the electron transport chain, ultimately reducing metmyoglobin localized near the mitochondrial outer membrane (a site of low oxygen partial pressure causing formation of metmyoglobin).

permeate through mitochondria (*36*), which makes malate a likely candidate for regeneration and transport of reducing equivalents to mitochondria by shuttle mechanisms that ultimately lead to MMb reduction. However, in post-mortem skeletal muscle, the role of malate in the malate−aspartate shuttle and its permeability to mitochondria is unknown. Future studies should examine the detailed physicochemical properties of malate and MDH and the efficacy of these biochemical pathways leading to myoglobin redox form stability.

Reduction of Metmyoglobin by Compartmentalized Lactate Dehydrogenase. Another purpose of this experiment was to assess the ability of compartmentalized LDH from isolated mitochondria and cytoplasm from muscles differing in color stability to use lactate as a substrate for NADH production and concomitant MMb reduction either directly or via the lactate−LDH system. Addition of lactate to isolated beef mitochondrial and cytoplasmic isolates resulted in MMb reduction via the lactate−LDH system. Mitochondria isolated from the three bovine muscles in this study readily oxidized lactate and presumably coupled with the upstream flow of electrons through the ETC and cytochrome *c*, resulting in the reduction of MMb (**Figure 9**). Brooks et al. (*37*) and Ramanathan et al. (*3*) reported similar observations of lactate oxidation where mitochondria isolated from bovine cardiac muscles had the capability of using exogenous lactate as an energy substrate, resulting in cellular respiration (*37*) and increased oxygen consumption (*3*). The density of mitochondria in cardiac muscle was related to lactate oxidation (*31*). Oxidation of lactate by bovine skeletal muscle mitochondria via a lactate−LDH system is consistent with previous reports (*3*, *16*−*18*, *38*) where lactate is an important substrate contributing to the pool of NADH and concomitant MMb reduction.

This study provides evidence that L-lactate added to isolated mitochondria and cytoplasmic protein is used as substrate for LDH, leading to regeneration of NADH and MMb reduction. The rate of MMb reduction was lower in the mitochondrial fraction than in the cytoplasmic fraction. Ability of mitochondrial

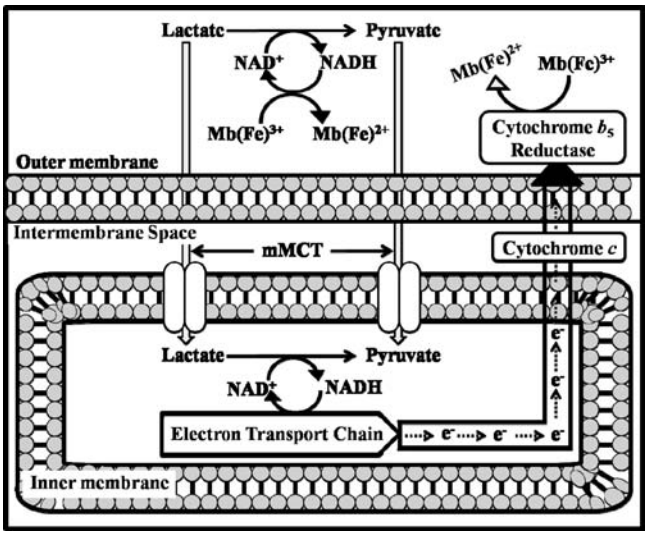

Figure 9. Proposed model and biochemical pathways for enzymatic and nonenzymatic reduction of metmyoglobin and depiction of the NADH production between cytoplasmic and mitochondrial LDH. Entry of lactate and pyruvate into the mitochondrial matrix is facilitated by mitochondrial monocarboxylate transporters (mMCT). Lactate is oxidized to pyruvate via mitochondrial LDH (m-LDH), regenerating the post-mortem pool of NADH with changes in mitochondrial redox potential, and the produced NADH is further oxidized via the electron transport chain, ultimately reducing metmyoglobin localized near the mitochondrial outer membrane (a site of low oxygen partial pressure causing formation of metmyoglobin).

LDH to convert lactate to pyruvate is more directly related to mitochondrial redox status than to the LDH isoenzyme pattern (*37*). To our knowledge, no previous study has provided evidence for compartmentalized LDH activity in post-mortem muscle with an ability to regenerate NADH and reduce MMb. The LDH activity of all muscles followed a different trend than that seen with m-MDH and c-MDH. The c-LDH-assisted MMb reduction was significantly higher for LL than PM, whereas that of ST was not significantly different from that of LL. Kim et al. (*38*) reported that a higher accumulation of MMb and lower MRA were associated with low LDH activity in PM and decreased NADH concentration post-mortem.

Influence of Post-Mortem Aging on Enzyme Activity and Distribution. Enzyme activity (LDH and MDH) and protein content (mitochondrial and cytoplasmic) in post-mortem aged meat were examined for relative decreases in enzyme activity. The aging times used in this study (10 and 20 days) represent typical industry practices and meat purchased by consumers. As the post-mortem aging period lengthens, enzymes are redistributed to new intracellular compartments. During post-mortem aging, proteolytic enzymes tend to become highly active, whereas some endogenous enzymes (cytosolic and mitochondrial) become more sensitive to oxidation or degradation.

Cytoplasmic and Mitochondrial Protein Content. Both cytoplasmic and mitochondrial proteins of the three muscles studied indicate important muscle-specific effects that could affect color stability. Aging from 10 to 20 days decreased both cytoplasmic protein and mitochondrial protein levels. PM had the highest levels of both cytoplasmic and mitochondrial proteins compared with LL and ST; however, aging decreased cytoplasmic and mitochondrial proteins in all muscles. It is possible that differences in estimates of mitochondrial and cytoplasmic protein content reported in this study are due to fiber type (slow and fast twitch) and the different metabolic activity of the three beef muscles discussed earlier. Beef skeletal muscles used as sources of muscle mitochondria in this study are composed of either type I or type IIb fibers.

Metmyoglobin-Reducing Activity Assay. Data for MRA corresponded well with m-MDH and c-MDH and/or LDH assays. As expected, muscles with greater color stability, such as LL and ST, had more MRA than PM, the more color unstable muscle, at both aging times (10 and 20 days). Our research documenting differences in muscles' ability to regenerate NADH and concomitantly reduce MMb by using cytoplasmic and mitochondrial enzymes and their agreement with the specific MRA assay adds a new dimension to knowledge of meat color stability. Researchers have documented different methods to quantify MRA; however, their findings for relative color stability differences among muscles are similar to those in the present study. Ledward (*39*) placed the PM and LL muscles into anaerobic environments after display and measured the extent of MMb reduction by the reflectance method. Reddy and Carpenter (*40*) reported that muscles traditionally characterized as the most color stable had higher reducing activities. Our data clearly show that the linkage between a muscle's ability to regenerate NADH and the subsequent MMb reduction are primary endogenous determinants of relative color stability.

Reddy and Carpenter (*40*) used muscle extracts, ferrocyanide, and horse MMb in solution with NADH to show that the LL had more MRA than ST and PM muscles. MRA assays used in the current study revealed that LL had more MRA than both ST and PM muscles but that MRA of the ST appeared to be more intermediate than that of the PM. Sammel et al. (*28*, *41*) used a MRA procedure similar to that used in the current study and reported that superficial portions of the *M. semimembranosus* had greater MRA and more color stability than deep portions. O'Keeffe and Hood (*42*) reported that MRA of LL, SM, and PM muscles decreased as storage time increased. Madhavi and Carpenter (*40*) reported similar findings and suggested that muscle oxygen consumption and its MRA have biphasic effects on color stability during aging. In that study, muscle oxygen consumption was the primary determinant of color stability during the first 7 days post-mortem, whereas MRA was most important after 7 days. Overall, measures of MRA in this study seemed reliable because they showed good agreement with CcOx activity and enzymatic reduction of MMb using cytoplasmic and mitochondrial proteins.

Cytochrome *c* Oxidase Activity. Differences in CcOx activity among the three muscles corresponded well with MRA activity and color stability differences exhibited by these muscles.

Furthermore, because mitochondria are considered as the principle site for MMb reduction and a source of NADH, a key component for MRA, CcOx activity within different muscles should relate to their reducing ability and the relative color stability differences. Seyfert et al. (*2*) assessed CcOx activity of five different bovine skeletal muscles and found that the superficial region (region of muscle adjacent to subcutaneous fat) of the *semimembranosus* muscle had twice as much CcOx activity as the deep (inner region of muscle closest to the femur) region.

In summary, our results suggest that the LL had almost twice as much CcOx activity as the PM. Also, beef muscles aged for 10 days had higher CcOx activity than muscles aged for 20 days. A difference in CcOx activity of beef skeletal muscles of varied color stability might explain color stability differences among LL, ST, and PM. Furthermore, the greater differences in CcOx activity of beef skeletal muscles due to aging time appear to be related; the 20 day aging period resulted in greater protein and enzymatic denaturation and/or oxidation. Aging beef muscles for another 20 days likely would have caused denaturation of enzymes within mitochondria, particularly CcOx, resulting in

decreased MRA. It is generally believed that reducing activity of individual muscles is the most important factor in determining rate of discoloration.

This study clearly indicates that mitochondria and cytoplasmic proteins isolated from beef skeletal muscles of different metabolic origin differ substantially in enzymatic composition. Malate−MDH-assisted MMb reduction using mitochondrial and cytoplasmic isolates from the three beef skeletal muscles exhibited substantial differences in enzymatic compositions and their ability to reduce MMb *in vitro*. Differences were also observed in enzymatic characteristics of MDH-assisted MMb reduction among the three beef muscles. This study further confirmed the presence of localized m-LDH and c-LDH and supports a role for MMb reduction via the lactate−LDH system. Our data clearly show that the linkage between a muscle's ability to regenerate NADH and the subsequent reduction of MMb is the primary endogenous determinant of relative color stability. Moreover, the finding that addition of malate and lactate in combination to mitochondrial and cytoplasmic isolates was completely additive in reducing MMb suggests an important role of glycolytic and mitochondrial TCA metabolites in the enzymatic reduction of MMb. Relative contributions of other factors, such as CcOx and MRA, may also play an important role in regulating myoglobin redox stability and ultimately determine the color stability of muscle.

ABBREVIATIONS USED

LL, *Longissimus lumborum*; PM, *Psoas major*; ST, *Semitendinosus*; BSA, bovine serum albumin; CcOx, cytochrome *c* oxidase; c-LDH, cytoplasmic LDH; c-MDH, cytoplasmic MDH; DMb, deoxymyoglobin; EDTA, ethylenediaminetetraacetic acid; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; m-MDH, mitochondrial MDH; m-LDH, mitochondrial LDH; MMb, metymyoglobin; MRA, metmyoglobin-reducing activity; NAD^+, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; TCA, tricarboxylic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

LITERATURE CITED

(1) Tang, J.; Faustman, C.; Mancini, R. A.; Seyfert, M.; Hunt, M. C. Mitochondrial reduction of metmyoglobin: Dependence on the electron transport chain. *J. Agric. Food Chem.* **2005**, *53*, 5449–5455.

(2) Seyfert, M.; Mancini, R. A.; Hunt, M. C.; Tang, J.; Faustman, C.; Garcia, M. Color stability, reducing activity, and cytochrome *c* oxidase activity of five bovine muscles. *J. Agric. Food Chem.* **2006**, *54*, 8919–8925.

(3) Ramanathan, R.; Mancini, R. A.; Konda, M. R. Effects of lactate on beef heart mitochondrial oxygen consumption and muscle darkening. *J. Agric. Food Chem.* **2009**, *57*, 1550–1555.

(4) Andrews, M. M.; Guthneck, B. T.; McBride, B. H.; Schweigert, B. S. Stability of certain respiratory and glycolytic enzyme systems in animal tissues. *J. Biol. Chem.* **1952**, *194*, 715–719.

(5) Bodwell, C.; Pearson, A.; Fennell, R. Post-mortem changes in muscle. III. Histochemical observations in beef and pork. *J. Food Sci.* **1965**, *30*, 944.

(6) Tang, J.; Faustman, C.; Hoagland, T. A.; Mancini, R. A.; Seyfert, M.; Hunt, M. C. Postmortem oxygen consumption by mitochondria and its effects on myoglobin form and stability. *J. Agric. Food Chem.* **2005**, *53*, 1223–1230.

(7) Mohan, A.; Muthukrishnan, S.; Hunt, M. C.; Barstow, T. J.; Houser, T. A. Kinetics of myoglobin redox form stabilization by malate dehydrogenase. *J. Agric. Food Chem.* **2010** (in press).

(8) Mohan, A.; Hunt, M. C.; Barstow, T. J.; Houser, T. A.; Muthukrishnan, S. Effects of malate, lactate, and pyruvate on myoglobin redox stability in homogenates of three bovine muscles. *Meat Sci.* **2010** (in press).

(9) Arihara, K.; Cassens, R. G.; Greaser, M. L.; Luchansky, J. B.; Mozdziak, P. E. Localization of metmyoglobin-reducing enzyme (NADH-cytochrome b_5 reductase) system components in bovine skeletal muscle. *Meat Sci.* **1995**, *39*, 205–213.

(10) Renerre, M. Variabilité entre muscles et entre animaux de la stabilité de la couleur des viandes bovines. *Sci. Aliments* **1984**, *4*, 567–584.

(11) Renerre, M.; Labas, R. Biochemical factors influencing metmyoglobin formation in beef muscles. *Meat Sci.* **1987**, *19*, 151–165.

(12) Lanari, M. C.; Cassens, R. G. Mitochondrial activity and beef muscle color stability. *J. Food Sci.* **1991**, *56*, 1476–1479.

(13) Giddings, G. G. Reduction of ferrimyoglobin in meat. *CRC Crit. Rev. Food Technol.* **1974**, *5*, 143–173.

(14) Klingenberg, M.; Bucher, T. Biological oxidations. *Annu. Rev. Biochem.* **1960**, *29*, 669–708.

(15) Brown, W. D.; Snyder, H. E. Nonenzymatic reduction and oxidation of myoglobin and hemoglobin by nicotinamide adenine dinucleotides and flavins. *J. Biol. Chem.* **1969**, *244*, 6702–6706.

(16) Mancini, R. A.; Hunt, M. C. Current research in meat color. *Meat Sci.* **2005**, *71*, 100–121.

(17) Kim, Y. H.; Hunt, M. C.; Mancini, R. A.; Seyfert, M.; Loughin, T. M.; Kropf, D. H.; Smith, J. S. Mechanism for lactate-color stabilization in injection-enhanced beef. *J. Agric. Food Chem.* **2006**, *54*, 7856–7862.

(18) Mancini, R. A.; Ramanathan, R. Potential roles of lactate, pyruvate, succinate, and citrate in beef color. *Proc. Am. Meat Sci. Assoc. U.S.A.* **2009**, 1–5.

(19) Watts, B. M.; Kendrick, J.; Zipser, M. W.; Hutchins, B.; Saleh, B. Enzymatic reducing pathways in meat. *J. Food Sci.* **1966**, *31*, 855–862.

(20) Saleh, B.; Watts, B. M. Substrates and intermediates in the enzymatic reduction of metmyoglobin in ground beef. *J. Food Sci.* **1968**, *33*, 353–358.

(21) Hagler, L.; Coppes, R. I.; Herman, R. H. Metmyoglobin reductase. Identification and purification of a reduced nicotinamide adenine dinucleotide-dependent enzyme from bovine heart which reduces metmyoglobin. *J. Biol. Chem.* **1979**, *254*, 6505–6514.

(22) Mikkelsen, A.; Juncher, D.; Skibsted, L. H. Metmyoglobin reductase activity in porcine M. *Longissimus dorsi* muscle. *Meat Sci.* **1999**, *51*, 155–161.

(23) Hunt, M.; Hedrick, H. Chemical, physical and sensory characteristics of bovine muscle from four quality groups. *J. Food Sci.* **1977**, *42*, 716–720.

(24) Thiex, N. Evaluation of analytical methods for the determination of moisture, crude protein, crude fat, and crude fiber in distillers dried grains with solubles. *J. AOAC Int.* **2009**, *92*, 61–73.

(25) Keeton, J. T.; Morris, C. A. Sarcoplasmic enzymes as potential indicators of heating endpoints in meat products. *J. Muscle Foods* **1996**, *7*, 323–334.

(26) Bhattacharya, S. K.; Thakar, J. H.; Johnson, P. L.; Shanklin, D. R. Isolation of skeletal muscle mitochondria from hamsters using an lonic medium containing ethylenediarninetetraacetic acid and Nagarse. *Anal. Biochem.* **1991**, *192*, 344–349.

(27) Frezza, C.; Cipolat, S.; Scorrano, L. Organelle isolation: Functional mitochondria from mouse liver, muscle and cultured filroblasts. *Nat. Protoc.* **2007**, *2*, 287–295.

(28) Sammel, L. M.; Hunt, M. C.; Kropf, D. H.; Hachmeister, K. A.; Johnson, D. E. Comparison of assays for metmyoglobin reducing ability in beef inside and outside semimembranosus muscle. *J. Food Sci.* **2002**, *67*, 978–984.

(29) AMSA. *Guidelines for meat color evaluation*; American Meat Science Association: Chicago, IL, 1991.

(30) Cheah, K. Comparative studies of the mitochondrial properties of *Longissimus dorsi* muscles of Pietrain and Large White pigs. *J. Sci. Food Agric.* **1973**, *24*, 51–61.

(31) Hunt, M.; Hedrick, H. Histochemical and histological characteristics of bovine muscles from four quality groups. *J. Food Sci.* **1977**, *42*, 578–582.

(32) Hoppeler, H.; Hudlicka, O.; Uhlmann, E. Relationship between mitochondria and oxygen consumption in isolated cat muscles. *J. Physiol.* (*London*) **1987**, *385*, 661–675.

(33) LaNoue, K. F.; Williamson, J. R. Interrelationships between malate-aspartate shuttle and citric acid cycle in rat heart mitochondria. *Metabolism* **1971**, *20*, 119–140.

(34) McElroy, F. A.; Wong, G. S.; Williams, G. R. Distribution of malate across the mitochondrial membrane as a signifacant factor in respiratory control. *Arch. Biochem. Biophys.* **1968**, *128*, 563–565.
(35) Markert, C. L.; Møller, F. Multiple forms of enzymes: Tissue, ontogenetic, and species specific patterns. *Proc. Natl. Acad. Sci. U.S.A.* **1959**, *45*, 753–763.
(36) Purvis, J. L.; Lowenstein, J. M. The relation between intra- and extramitochondrial pyridine nucleotides. *J. Biol. Chem.* **1961**, *236*, 2794–2803.
(37) Brooks, G. A.; Dubouchaud, H.; Brown, M.; Sicurello, J. P.; Butz, C. E. Role of mitochondrial lactate dehydrogenase and lactate oxidation in the intracellular lactate shuttle. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1129–1134.
(38) Kim, Y. H.; Keeton, J. T.; Smith, S. B.; Maxim, J. E.; Yang, H. S.; Savell, J. W. Evaluation of antioxidant capacity and color stability of calcium lactate enhancement on fresh beef under highly oxidizing conditions. *Food Chem.* **2009**, *115*, 272–278.
(39) Ledward, D. A. Post-slaughter influences on the formation of metmyoglobin in beef muscles. *Meat Sci.* **1985**, *15*, 149–171.
(40) Reddy, L. M.; Carpenter, C. E. Determination of metmyoglobin reductase activity in bovine skeletal muscles. *J. Food Sci.* **1991**, *56*, 1161–1164.
(41) Sammel, L. M.; Hunt, M. C.; Kropf, D. H.; Hachmeister, K. A.; Kastner, C. L.; Johnson, D. E. Influence of chemical characteristics of beef inside and outside *semimembranosus* on color traits. *J. Food Sci.* **2002**, *67*, 1323–1330.
(42) O'Keeffe, M.; Hood, D. Biochemical factors influencing metmyoglobin formation on beef from muscles of differing color stability. *Meat Sci.* **1982**, *7*, 209–228.

Received for review February 21, 2010. Revised manuscript received April 24, 2010. Accepted April 27, 2010. Contribution No. 10-204-J from the Kansas Agricultural Experiment Station, Manhattan, KS 66506.