JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Postmortem Oxygen Consumption by Mitochondria and Its Effects on Myoglobin Form and Stability

JIALI TANG,† CAMERON FAUSTMAN,*,† THOMAS A. HOAGLAND,† RICHARD A. MANCINI,† MARK SEYFERT,§ AND MELVIN C. HUNT§

Department of Animal Science, University of Connecticut, 3636 Horsebarn Hill Road Ext., Storrs, Connecticut 06269-4040, and Department of Animal Science and Industry, 224 Weber Hall, Kansas State University, Manhattan, Kansas 66506

The objective of this study was to assess the morphological integrity and functional potential of mitochondria from postmortem bovine cardiac muscle and evaluate mitochondrial interactions with myoglobin (Mb) in vitro. Electron microscopy revealed that mitochondria maintained structural integrity at 2 h postmortem; prolonged storage resulted in swelling and breakage. At 2 h, 96 h, and 60 days postmortem, the mitochondrial state III oxygen consumption rate (OCR) and respiratory control ratio decreased with time at pH 7.2 and 5.6 ($p < 0.05$). Mitochondria isolated at 60 days did not exhibit ADP-induced transitions from state IV to state III oxygen consumption. Tissue oxygen consumption also decreased with time postmortem ($p < 0.05$). Mitochondrial oxygen consumption was inhibited by decreased pH in vitro ($p < 0.05$). In a closed system, mitochondrial respiration resulted in decreased oxygen partial pressure (pO_2) and enhanced conversion of oxymyoglobin (OxyMb) to deoxymyoglobin (DeoMb) or metmyoglobin (MetMb). Greater mitochondrial densities caused rapid decreases in pO_2 and favored DeoMb formation at pH 7.2 in closed systems ($p < 0.05$); there was no effect on MetMb formation ($p > 0.05$). MetMb formation was inversely proportional to mitochondrial density at pH 5.6 in closed systems. Mitochondrial respiration in open systems resulted in greater MetMb and DeoMb formation at pH 5.6 and pH 7.2, respectively, vs controls ($p < 0.05$). The greatest MetMb formation was observed with a mitochondrial density of 0.5 mg/mL at both pH values in open systems. Mitochondrial respiration facilitated a shift in Mb form from OxyMb to DeoMb or MetMb, and this was dependent on pH, oxygen availability, and mitochondrial density.

KEYWORDS: Mitochondria; myoglobin; oxygen consumption rate; partial oxygen pressure

INTRODUCTION

Fresh meat color is determined by the relative proportions of the three forms of myoglobin (Mb): deoxymyoglobin (DeoMb), oxymyoglobin (OxyMb), and metmyoglobin (MetMb). DeoMb is purplish in color, and OxyMb is bright red in color. Oxidation of DeoMb or OxyMb produces brownish MetMb. Binding of oxygen to DeoMb causes the formation of OxyMb, which is responsible for the consumer-preferred color of fresh meat. Interconversion among DeoMb, OxyMb, and MetMb is influenced by several factors including pH, temperature, relative humidity, partial oxygen pressure (pO_2), MetMb reducing activity, and lipid oxidation (*1*).

Mitochondria are important subcellular organelles involved in energy metabolism. Mb and mitochondria are interrelated in living cells as Mb serves as an oxygen reservoir and oxygen transporter for mitochondria. These subcellular organelles use >80% of the oxygen available in cell cytoplasm (*2*−*5*). Changes in structure and function of mitochondria in postmortem muscle were first reported by Cheah et al. (*6*, *7*). Mitochondria isolated at 0.5 and 96 h postmortem, all in the condensed configuration, had clearly defined outer and inner membranes and distinct and closely packed cristae; the authors observed a small proportion of swollen mitochondria at 120 and 144 h postmortem (*7*). Intact mitochondria were also isolated from ox neck muscle after storage in situ for up to a maximum of 10 days at 2 °C (*6*). Mitochondrial (MT) functional ability also decreased with time postmortem when malate plus pyruvate, succinate, or ascorbate plus tetramethyl-*p*-phenylenediamine were used as substrates at pH 7.2 and 25 °C. Mitochondria from tissue obtained 96 h postmortem retained 70% of initial values (0.5 h tissue) for state III oxygen consumption rate (OCR) with all of these substrates (*7*). The effects of prolonged storage on MT morphology, functional integrity, and oxygen consumption at the ultimate pH (5.6) of meat have not been reported.

Although evidence for a direct interaction between mitochondria and Mb is lacking, some observations have been reported that relate postmortem muscle oxygen consumption,

* To whom correspondence should be addressed. Tel: 860-486-2413. Fax: 860-486-4375. E-mail: cameron.faustman@uconn.edu.
† University of Connecticut.
§ Kansas State University.

10.1021/jf048646o CCC: $30.25
Published on Web 01/22/2005

presumably mediated by mitochondria, to meat color. *Longissimus dorsi* and *gluteus medius* muscles from Holstein cattle demonstrated greater OCR than did muscles of crossbred animals (*8*). The retail display life of meat was inversely proportional to the log oxygen uptake when lamb, pork, and beef *semimembranosus* muscles were evaluated. Beef muscle demonstrated the lowest levels of oxygen uptake and the longest display life, while lamb muscle had the greatest oxygen uptake and shortest display life (*9*). Color labile muscles, such as *psoas major* or *diaphragma medialis*, had greater muscle OCR than color stable muscles, such as *longissimus dorsi* (*10*−*12*). Lanari et al. (*8*) further related beef muscle color stability to MT content and OCR and found that muscle of lower color stability (*gluteus medius*) had a relatively greater MT density and OCR postmortem as compared with the more color stable *longissimus dorsi*. Treatment of prerigor or dark cutting beef muscle homogenates with rotenone, lower pH, or lower temperature in high oxygen environments appeared to decrease MT respiration and improve Mb oxygenation and meat color (*13*−*15*).

Collectively, these observations suggest that mitochondria can play an important role in meat color stability. The objective of this study was to assess the morphological integrity and functional potential of mitochondria from postmortem bovine cardiac muscle at pH 5.6 and investigate specifically the potential interactions between mitochondria and Mb in vitro.

MATERIALS AND METHODS

Materials and Chemicals. Beef hearts were obtained locally within 0.5 h of exsanguination, placed on ice, and transported to the laboratory; samples for analysis at 96 h and 60 days were vacuum-packaged and stored at 4 °C. Equine heart Mb, sodium hydrosulfite, magnesium chloride ($MgCl_2$), bovine albumin (BSA), sucrose, Tris[hydroxymethyl]-aminomethane hydrochloride (Tris-HCl), ethylenediaminetetraacetic acid (EDTA), potassium phosphate monobasic (KH_2PO_4), ethylene glycolbis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), 2-[*N*-morpholino]ethanesulfonic acid (MES), maleic acid, adenosine 5′-diphosphate (ADP), 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 reagent grade.

Equine OxyMb Preparation. OxyMb was prepared according to Brown and Mebine (*16*) as follows. Commercial Mb (Sigma Chemical Co.) was dissolved in 50 mM Tris-HCl buffer (pH 7.4) and chemically reduced by mixing with sodium hydrosulfite. Air was bubbled through solution to oxygenate reduced Mb, and the residual sodium hydrosulfite was removed by dialysis against 50 mM Tris-HCl buffer.

Bovine Mitochondria Isolation. Mitochondria were isolated from bovine cardiac muscle according to Smith (*17*) with minor modifications. Briefly, 100 g of ground cardiac muscle was washed with 250 mM sucrose twice and suspended into 200 mL of MT isolation buffer (250 mM sucrose, 10 mM HEPES, 1 mM EGTA, and 0.1% BSA, pH 7.4). The suspension was stirred slowly and hydrolyzed with Nagarse protease (protease/tissue = 0.5 mg/g) for 20 min; the pH was maintained between 7.0 and 7.4. After proteolytic digestion, the suspension was diluted to 1000 mL with MT isolation buffer and subjected to two homogenization processes. The first of these was accomplished in a Kontes Duall grinder (Vineland, NJ) with three passes and was followed by treatment with a Wheaton Potter-Elvehjem grinder (Millville, NJ) with one pass; pestles of these grinders were driven by a heavy duty drill at 1400 rpm. The homogenate was centrifuged (1200*g*) for 20 min with a Sorvall RC-5B centrifuge (Newtown, CT), and the resulting supernatant was then centrifuged (26000*g*) for 15 min. The pellet was washed twice and suspended in MT suspension buffer (250 mM sucrose, 10 mM HEPES, pH 7.4) and was ready for use. All steps were performed at 0−4 °C. The MT protein content was determined by a Bicinchoninic Acid Protein Assay Kit from Sigma Chemical Co.

Oxygen Consumption Measurement. MT oxygen uptake was measured with a Clark oxygen electrode using a polarizing voltage of 0.60 V, which was clamped to the bottom of an 8 mL incubation chamber. Reaction components were added to the chamber, and the temperature was maintained at 25 °C by a water jacket and Lauda RE120 circulating water bath (Westbury, NY). The chamber was stirred with a 10 mm Teflon-covered bar at 600 rpm. Additions were made with Hamilton syringes through a 1 mm port. The electrode was attached to a Rank Brothers digital model 20 oxygen controller (Cambridge, England) and connected to a personal computer with data logger. Oxygen consumption was recorded over time at pH 7.2 (250 mM sucrose, 5 mM KH_2PO_4, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% BSA, and 20 mM HEPES), 6.4 (same as pH 7.2 except MES replaced HEPES), or 5.6 (same as pH 7.2 except maleic acid replaced HEPES). State IV OCR is the OCR of isolated mitochondria in the presence of substrate, while state III OCR is the OCR of isolated mitochondria in the presence of both substrate and ADP. The respiratory control ratio (RCR) is the ratio between state III OCR and state IV OCR, which is an indication of integrity of isolated mitochondria. Calculations for state III and IV OCR, RCR, and the ratio of ADP to oxygen consumption during phosphorylation (ADP/O) were based on the method of Estabrook (*18*). The Clark oxygen electrode was also used for measuring tissue oxygen consumption. The tissue was ground and added (0.1 g/mL) to pH 7.2 (120 mM KCl, 30 mM HEPES) or 5.6 (120 mM KCl, 30 mM maleic acid) buffers, and OCR was then measured without addition of any substrate.

Sample Preparation for Electron Microscopy. The MT pellet resulting from differential centrifugation or strips of cardiac muscle (10 mm × 2 mm × 0.5 mm) were fixed in 1.5% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M HEPES buffer with 3 mM $CaCl_2$, pH 7.3, for approximately 1.5 h. Samples were cut into 0.5−1.0 mm cubes, washed in buffer overnight, postfixed the following morning in 1% osmium tetroxide and 0.8% potassium ferricyanide in 0.1 M HEPES buffer with 3 mM $CaCl_2$ for 1 h, dehydrated in a graded ethanol series, and embedded in Eponate 812. All steps prior to dehydration with 70% ethanol were performed at 4 °C; those thereafter were performed at room temperature. Sections were cut on a diamond knife and stained with ethanolic uranyl acetate and lead citrate.

Postmortem Tissue pH Determination. The postmortem tissue pH was determined based on Bendall's method (*19*). Briefly, sodium iodoacetate (solution A, 0.1 M) and KCl (solution B, 1.5 M) solutions were prepared, and 20 mL of solution A was mixed with 40 mL of solution B and diluted to 400 mL just before use. Muscle (0.5 g) was added to 7 mL of mixed solution and homogenized using a Waring blender (New Hartford, CT) with high speed. The pH of the resulting homogenate was measured with a Fisher Accumet model 10 pH meter (Pittsburgh, PA).

Lipid Oxidation. Lipid oxidation in samples was determined by measuring thiobarbituric reactive substances according to Schmedes and Holmer (*20*). Briefly, 0.5 mL of sample was mixed with 0.5 mL of 11% trichloroacetic acid solution and then centrifuged for 3 min (15000*g*) with an Eppendorf 5415D centrifuge (Westbury, NY). The resulting supernatant (0.5 mL) was combined with 0.5 mL of 20 mM thiobarbituric acid and incubated at 25 °C for 20 h. The absorbance at 532 nm was measured with a Shimadzu UV 2101-PC (Kyoto, Japan) spectrophotometer.

Mitochondria and Mb Interaction in Closed Systems. Mitochondria (0.5 or 1.0 mg MT protein/mL, isolated 2 h postmortem) and Mb (0.15 mM) were combined with pH 7.2 (250 mM sucrose, 5 mM KH_2PO_4, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% BSA, and 20 mM HEPES) or 5.6 (same as pH 7.2 except maleic acid replaced HEPES) buffers in the incubation chamber to a final volume of 3 mL. This incubation chamber was identified as a "closed system" because it did not contain any headspace; thus, the potential diffusion of air from outside of the vessel into the solution was prevented. The starting O_2 concentration of the solution was taken as 100%, and the reaction was initiated with the addition of 8 mM succinate. At specific time and/or % O_2 intervals, samples were removed from the chamber and scanned from 650 to 500 nm with a Shimadzu UV-2101PC spectrophotometer with integrating sphere assembly. Solutions containing all components except Mb were used as blanks. DeoMb, OxyMb, and MetMb were calculated

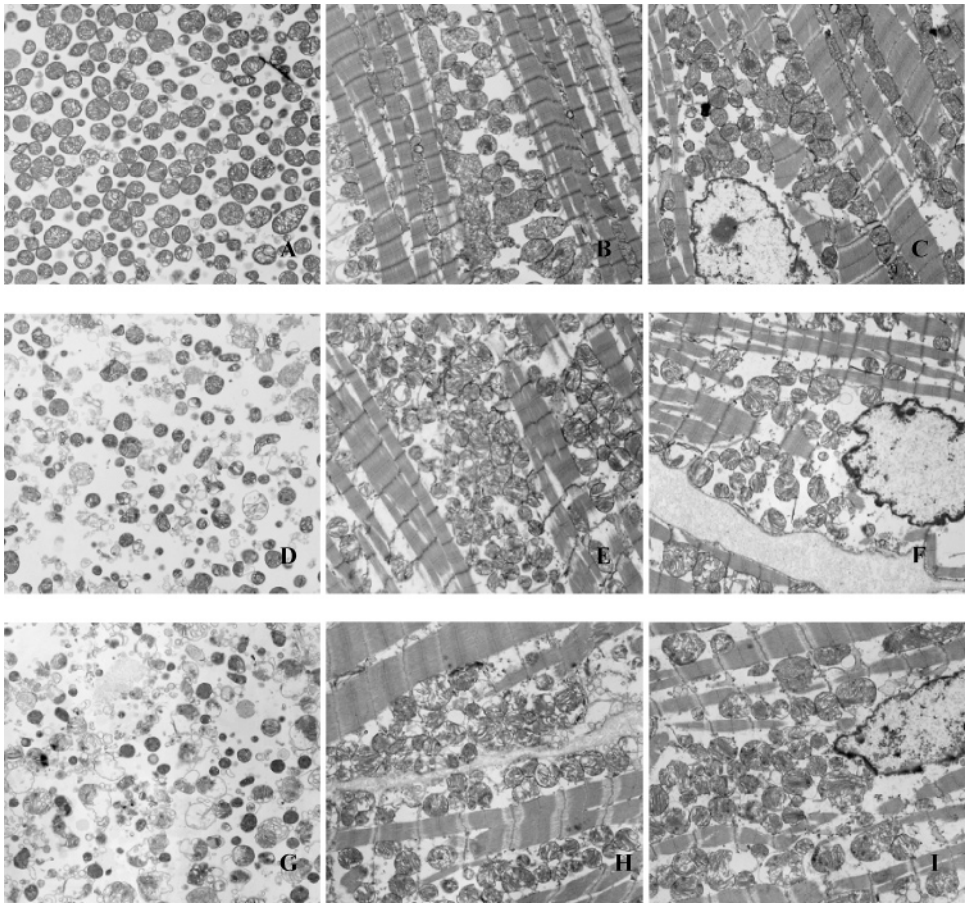

Figure 1. Mitochondria isolated from beef cardiac muscle at 2 h (**A**), 96 h (**D**), and 60 days (**G**); in situ intermyofibrillar mitochondria at 2 h (**B**), 96 h (**E**), and 60 days (**H**); or perinuclear mitochondria at 2 h (**C**), 96 h (**F**), and 60 days (**I**). Magnification: 5400×.

with the following equations, modified from Krzywicki's formulas (*21*, *22*). [DeoMb] = $-0.543R_1 + 1.594R_2 + 0.552R_3 - 1.329$, [OxyMb] = $0.722R_1 - 1.432R_2 - 1.659R_3 + 2.599$, and [MetMb] = $-0.159R_1 - 0.085R_2 + 1.262R_3 - 0.520$; $R_1 = A_{582}/A_{525}$; $R_2 = A_{557}/A_{525}$; and $R_3 = A_{503}/A_{525}$.

Mitochondria and Mb Interaction in Open Systems. The mitochondria and Mb interaction was also investigated in a system that permitted air to diffuse freely into the suspension and was referred to as an "open system"; no oxygen consumption measurement was possible with this approach. Control (0.15 mM Mb, 1 mg MT protein/mL, isolated 2 h postmortem) and treatment (control plus 8 mM succinate) samples were prepared with pH 5.6 (120 mM KCl, 30 mM maleic acid, and 5 mM KH_2PO_4) and 7.2 (120 mM KCl, 30 mM Tris-HCl, and 5 mM KH_2PO_4) buffers. One milliliter of sample was added to a 1.5 mL microfuge open-top tube and incubated at 25 °C. The samples were removed hourly for scanning spectrophotometry. The effect of MT concentration (0.1, 0.5, and 1.0 mg protein/mL) on Mb form and stability was also evaluated in this open system at pH 5.6 and 7.2 and 25 °C.

Statistical Analysis. Results are expressed as mean values (±SE) of three independent trials. Data were analyzed using the GLM procedure of SAS, and differences among means were detected at the 5% level using least significance difference with appropriate correction for multiple comparisons (*23*).

RESULTS AND DISCUSSION

MT Morphology and Function. Electron microscopic examination revealed intact mitochondria from both in vitro and in situ samples at 2 h postmortem; they were in a condensed configuration and demonstrated clear outer membranes (**Figure 1A−C**), similar to reports by Cheah et al. (*7*). After 96 h postmortem, most mitochondria demonstrated morphological changes with visible fragments in vitro; swollen mitochondria with large vacuoles were readily observed in situ (**Figure 1D−F**). Cheah et al. (*7*) reported that mitochondria obtained from ox neck muscle 96 h postmortem were still intact. The apparent degradation of MT integrity in our study may be related to the more rapid pH drop that we observed in cardiac muscle. Only 2 h was required for beef cardiac muscle to reach ultimate pH (**Table 1**), while Cheah et al. (*7*) reported that more than 48 h was required for ultimate pH to be achieved in ox neck muscle. Differences in MT morphology between 96 h and 60 days for both in vitro and in situ samples were not readily apparent (**Figure 1D−I**). The appearance of fragments in samples of isolated mitochondria at 96 h and 60 days may have resulted from increased fragility and susceptibility to damage by isolation procedures that occurred with time.

Table 1. Postmortem Cardiac Muscle pH ($n = 3$)

time postmortem (h)	pH	time postmortem (h)	pH
0.5	6.01 ± 0.07	8.0	5.82 ± 0.01
1.5	5.87 ± 0.02	24.0	5.84 ± 0.03
2.0	5.80 ± 0.02	72.0	5.83 ± 0.01
5.0	5.79 ± 0.02	120.0	5.83 ± 0.02

The metabolic activity of isolated mitochondria decreased from 2 to 96 h postmortem (**Table 2**). MT state III and IV OCR decreased during this period at both pH 7.2 and 5.6 ($p < 0.05$); 96 h state III OCR at both pH values was 60% that of mitochondria isolated at 2 h postmortem (**Table 2**). RCR also decreased following time postmortem at each incubation pH although the effect was not significant from 2 to 96 h ($p >$

Table 2. Tissue OCRs, MT State III and IV OCR (nmol O/min mg MT Protein or Tissue), RCR, and ADP/O[a] ($n = 3$)

pH	time	state III OCR	state IV OCR	RCR	ADP/O	tissue OCR
7.2	2 h	164.4 ± 1.5 a[b]	46.3 ± 1.9 b	3.56 ± 0.15 a	1.63 ± 0.09	1.28 ± 0.18 a
	96 h	108.8 ± 3.8 b	35.0 ± 1.3 c	3.12 ± 0.20 a	1.39 ± 0.08	1.16 ± 0.15 a
	60 days	75.4 ± 0.6 c	75.4 ± 0.6 a	1.00 ± 0.00 b	N/A[c]	0.13 ± 0.02 b
5.6	2 h	85.6 ± 7.6 a	40.0 ± 2.3 a	2.14 ± 0.08 a	1.31 ± 0.12	0.41 ± 0.02 a
	96 h	51.2 ± 2.1 b	24.9 ± 0.8 b	2.06 ± 0.12 a	1.39 ± 0.07	0.29 ± 0.01 b
	60 days	22.4 ± 0.4 c	22.4 ± 0.4 b	1.00 ± 0.00 b	N/A	0.06 ± 0.00 c

[a] Mitochondria or tissue were obtained at 2 h, 96 h, and 60 days postmortem and assessed at pH 7.2 or 5.6 and 25 °C. State III OCR is the OCR of isolated mitochondria in the presence of substrate and ADP; state IV OCR is the OCR of isolated mitochondria in the presence of only substrate. RCR is the ratio between state III OCR and state IV OCR. ADP/O is the ratio between ADP and oxygen consumed by mitochondria during phosphorylation. [b] Values in columns with different letters are different for each incubation pH ($p < 0.05$). [c] Not available.

0.05; **Table 2**). These results suggested that the functional integrity of mitochondria postmortem was compromised but not eliminated. Mitochondria isolated at 60 days postmortem still had the capacity to consume oxygen albeit at much lower rates when compared with mitochondria isolated at an earlier time. State III OCR at both pH values greatly decreased when compared with mitochondria obtained at 96 h ($p < 0.05$; **Table 2**). Tsvetkov et al. (*24*) and Petrenko (*25*) reported that freeze−thaw treatment of fresh mitochondria, which ruptured membranes and decreased RCR from 4.7 to 1.2, increased state IV OCR 3.5× at pH 7.5 when compared with untreated mitochondria. These observations of metabolic change were attributed to the loss of coupling between substrate oxidation and ATP synthesis. Similar effects were observed in our work with mitochondria isolated at 96 h and 60 days. RCR of these mitochondria decreased from 3.12 to 1.00, and state IV OCR increased from 35.0 to 75.4 nmol O/min mg MT protein at pH 7.2. Decreases in state IV OCR at pH 5.6 between 96 h and 60 days were not significant ($p > 0.05$; **Table 2**). This result is in agreement with Lanari et al. (*8*) who reported no time effect on MT state IV OCR beyond 48 h with succinate as the substrate. Tissue OCR also decreased with time postmortem ($p < 0.05$; **Table 2**), and the decrease was relatively faster than state III and IV OCR for isolated mitochondria. Tissue OCR at 60 days was only 10−15% of that at 2 h postmortem when measured at pH 7.2 or 5.6. This appeared to be related to a lack of appropriate substrate for tissue at 60 days as subsequent addition of succinate stimulated tissue oxygen consumption (results not shown), a result consistent with reports by Andrews et al. (*26*) and Bodwell et al. (*27*). Both of these groups proposed that the lack of available substrates, rather than instability of specific enzyme systems in intact muscle tissue, was the factor limiting the metabolism of muscle tissue after storage. Most enzymes, including electron transport chain enzymes, were still active after 4 weeks postmortem, while major substrates were present at very low concentrations.

MT metabolic potential was also greatly inhibited by decreased pH. Most measured variables at equivalent time points were less robust at pH 5.6 than pH 7.2 ($p < 0.05$; **Table 2**). State III and IV OCR decreased with decreased pH ($p < 0.05$; **Figure 2**); no difference in RCR was observed from pH 7.2 to 6.4 ($p > 0.05$), while a substantial decrease occurred from 6.4 to 5.6 ($p < 0.05$; **Figure 2**). MT activity could be expected to be greater in dark, firm, and dry beef (DFD or dark cutting beef) than normal beef because of the higher ultimate pH associated with this meat quality anomaly. Ashmore et al. (*28*, *29*) compared MT activity from normal beef and DFD beef produced by subcutaneous injection of cattle with epinephrine 24 h prior to slaughter. Mitochondria did not exhibit any differences in functional ability when they were isolated from normal or DFD fresh muscles (0 h after slaughter); however,

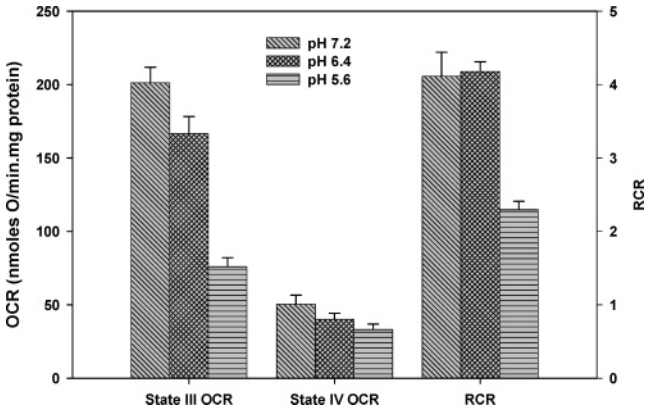

Figure 2. MT state III and IV OCRs (nmol O/min mg MT protein) and RCR at pH 7.2, 6.4, and 5.6 and 25 °C. State III OCR is the OCR of isolated mitochondria in the presence of substrate and ADP; state IV OCR is the OCR of isolated mitochondria in the presence of only substrate. RCR is the ratio between state III OCR and state IV OCR. Standard error bars are indicated ($n = 3$).

mitochondria isolated from DFD beef after 24 h postmortem were more active than those from normal beef when assessed at pH 6.7 and 5.7. Greater oxygen consumption from MT activity has been suggested to be responsible for the dark color of DFD meat presumably because active respiration causes OxyMb to release oxygen and change to DeoMb (*30*). Practices, such as injecting lactic acid or slow freeze−thaw treatment of dark cutting beef, were proposed as ways to decrease MT activity and stimulate Mb oxygenation; however, both methods failed to produce the hypothesized results (*15*). The lack of effect by lactic acid could be related to nonhomogeneous distribution of the lactic acid solution within muscle; freeze−thaw treatment might even increase oxygen consumption especially at higher pH values as reported previously (*24*, *25*).

In general, mitochondria isolated up to 60 days postmortem still had the capacity to consume oxygen despite observed compromised morphological integrity; the functional ability was decreased with time postmortem and decreased pH.

Mitochondria and Myoglobin Interactions. Interactions between mitochondria and myoglobin were investigated in vitro under different experimental conditions of pH, oxygen availability, and MT concentration. ADP was not included in the experiments because of its rapid breakdown in prerigor postmortem tissue. The effect of MT respiration on lipid oxidation is unsolved. Gabbita et al. (*5*) reported that respiratory stimulation of mitochondria, due to a hypermetabolic stress with succinate (20 mM) under state IV condition, caused significant oxidative modification of cortical membrane lipids and proteins. Other authors suggested that succinate-induced MT respiration inhibited NAD(P)H-ADP-Fe^{3+}-dependent lipid oxidation and

Table 3. Changes in O_2 Concentration and Mb Form (%) As Affected by MT Concentration at pH 5.6 and 25 °C in Closed Systems[a] ($n = 3$)

MT (mg/mL)	O_2 (%)	time (min)	MetMb	DeoMb	OxyMb	DeoMb/MetMb
0.5	100	0 a[b]	14.8 ± 1.4 a	4.7 ± 0.3 a	80.3 ± 1.5 a	0.32 ± 0.03 a
	15.00	20.8 ± 1.6 d	24.8 ± 1.0 d	7.1 ± 0.3 a	68.1 ± 0.8 bc	0.29 ± 0.02 a
	6.00	25.7 ± 2.0 e	25.3 ± 2.0 d	11.0 ± 1.5 ab	63.8 ± 0.7 c	0.45 ± 0.09 b
	2.00	29.1 ± 2.2 ef	30.9 ± 2.6 e	24.3 ± 1.6 c	45.0 ± 2.1 d	0.80 ± 0.11 d
	0.50	32.6 ± 1.9 f	36.4 ± 2.2 f	44.6 ± 4.9 d	19.1 ± 3.0 e	1.25 ± 0.19 e
1.0	100	0 a	16.1 ± 1.4 ab	5.2 ± 0.5 a	78.4 ± 0.8 a	0.33 ± 0.06 a
	15.00	12.0 ± 2.5 b	19.4 ± 2.6 bc	7.9 ± 1.0 a	72.8 ± 3.4 ab	0.41 ± 0.02 ab
	6.00	12.7 ± 2.4 b	20.2 ± 2.2 c	14.0 ± 1.4 b	65.8 ± 3.3 c	0.70 ± 0.04 c
	2.00	14.5 ± 2.4 bc	23.9 ± 2.4 d	29.1 ± 2.7 c	47.1 ± 4.2 d	1.24 ± 0.13 e
	0.50	18.1 ± 3.7 cd	30.5 ± 0.9 e	56.6 ± 4.0 e	13.0 ± 4.7 e	1.85 ± 0.10 f

[a] Incubation medium contained 250 mM sucrose, 5 mM KH_2PO_4, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% BSA, 20 mM maleic acid, 8 mM succinate, 0.15 mM Mb, and 0.5 or 1 mg MT protein/mL. [b] Values in columns with different letters are different ($p < 0.05$).

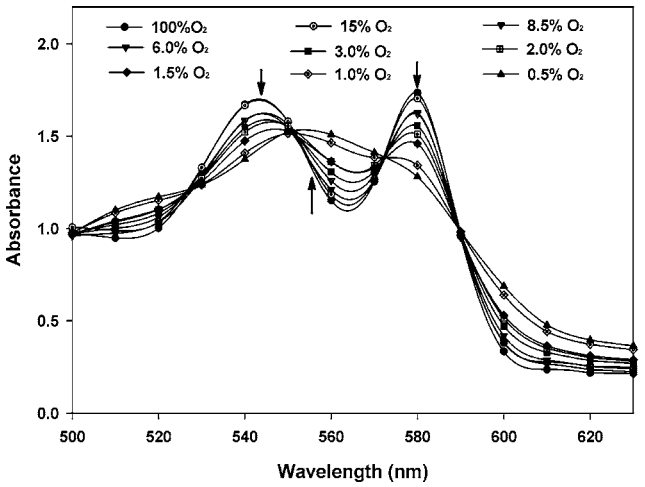

Figure 3. Mb spectra as affected by $\%O_2$ resulting from continuous MT respiration in a closed system at 25 °C and pH 7.2. The systems contained MT (1 mg MT protein/mL), Mb (0.15 mM), and succinate (8 mM); oxygen concentration was decreased from 100 to 0.5% in 12.3 min. Arrows indicate the direction of change in absorbance that occurred with MT oxygen consumption during the time course of the experiment.

malondialdehyde formation (*31*−*33*). Lipid oxidation was also monitored in our reaction systems as products of this breakdown process have been shown to bind to OxyMb and increase MetMb formation (*34*−*36*); however, no lipid oxidation was observed at either pH investigated in our reaction systems (results not shown).

Mitochondria and OxyMb were combined in a closed system at pH 7.2 and 25 °C, and the reaction was initiated by addition of succinate. As pO_2 decreased with MT respiration, the absorbance at 582 and 544 nm decreased, and absorbance increased at 557 nm (**Figure 3**), which are results that are consistent with decreased OxyMb and increased DeoMb concentrations. A spectrum typical of DeoMb was observed when pO_2 reached 0.5% of its initial value (**Figure 3**). A similar result was observed at pH 5.6 and 25 °C (results not shown), but more time was required to generate a typical DeoMb spectrum because mitochondria appeared less metabolically active at this lower pH. These results demonstrated directly that MT respiration influenced Mb form and stability by decreasing pO_2. George et al. (*37*, *38*) reported that the effect of pO_2 on Mb oxidation at pH 5.7 and 30 °C was first order over a range of 0.3−760 mmHg. The first order rate constant increased with increasing pO_2 with a maximal value at 1−1.4 mmHg, which is approximately the same pressure required for DeoMb half-saturation (P_{50}), and then decreased to a constant value above 30 mmHg. The results in **Tables 3** and **4** demonstrated that average MetMb formation rates (e.g., 2.8 vs 0.3 μmol/min at pH 5.6 and 1 mg MT protein/mL) were greater in the range of 15 to 0.5% O_2 ($pO_2 = 24$ to 0.8 mmHg at 1 atm) than from 100 to 15% O_2 ($pO_2 = 159$ to 24 mmHg) at both pH values and MT densities. Wazawa (*39*) offered a potential explanation for this result. He suggested that hydrogen peroxide played a key role in pO_2-dependent effects on Mb oxidation rates. Under air-saturated conditions, OxyMb autoxidation produced MetMb and superoxide anion (O_2^-), O_2^- was converted to H_2O_2 by dismutation and subsequently decomposed by MetMb; thus, MetMb resulted primarily from OxyMb autoxidation. However, at lower pO_2 values, DeoMb concentration increased with decreasing pO_2; H_2O_2 from OxyMb autoxidation reacted rapidly with DeoMb to form MetMb, resulting in both OxyMb autoxidation and H_2O_2-induced DeoMb oxidation contributing to MetMb formation. In the present study, DeoMb concentration began to increase when O_2 concentration decreased to 15% or lower ($pO_2 = 24$ mmHg at 1 atm; **Tables 3** and **4**).

The effect of MT density on O_2 concentration and changes in the relative proportions of Mb redox forms is presented in **Tables 3** and **4**. Greater MT densities caused rapid decreases in oxygen concentration at both pH values ($p < 0.05$; **Tables 3** and **4**). At pH 5.6 and for a given O_2 concentration, the MetMb formation was greater at lower MT densities than at higher densities ($p < 0.05$; **Table 3**), and it appeared that a rapid decrease in O_2 concentration was responsible for less MetMb formation in more densely concentrated samples. The time required to decrease O_2 concentration from 100 to 0.5% for more dense samples was 18.1 min, while at lower densities it was 32.6 min. At pH 7.2, the DeoMb formation was greater at greater MT densities when the oxygen concentration was 6.0% or lower ($p < 0.05$; **Table 4**), while no differences were observed in the MetMb formation ($p > 0.05$; **Table 4**). For a given O_2 concentration, the ratio of DeoMb to MetMb was greater with greater MT densities at both pH values ($p < 0.05$; **Tables 3** and **4**). These results suggested that rapid decreases in pO_2 maintained Mb in the DeoMb form.

Mitochondria and myoglobin interactions were also assessed in open systems at pH 7.2 and 5.6 and 25 °C. MT respiration accelerated the transition of OxyMb to DeoMb at all times and inhibited MetMb formation after 3 h at pH 7.2 when compared to controls (i.e., no succinate) ($p < 0.05$; **Figure 4**). Further study is needed to clarify the mechanism that is responsible for the inhibitory effect. At pH 5.6, MT respiration enhanced MetMb formation ($p < 0.05$; **Figure 4**), while no effect was observed on the DeoMb formation except at 1 h incubation, when compared with controls (**Figure 4**). Shikama (*40*) reported that OxyMb autoxidation was pH-dependent and the rate constant was greater at acidic pH than at physiological pH.

Table 4. Changes in O_2 Concentration and Mb Form (%) As Affected by MT Concentration at pH 7.2 and 25 °C in Closed Systems[a] ($n = 3$)

MT (mg/mL)	O_2 (%)	time (min)	MetMb	DeoMb	OxyMb	DeoMb/MetMb
0.5	100	0 a[b]	19.6 ± 0.8 a	5.0 ± 1.1 a	75.6 ± 1.6 a	0.25 ± 0.05 a
	15.00	10.6 ± 0.5 cd	21.7 ± 0.5 abc	9.7 ± 0.3 ab	68.8 ± 0.5 abc	0.45 ± 0.02 ab
	6.00	12.7 ± 0.7 de	22.3 ± 0.7 abc	14.6 ± 0.8 b	63.1 ± 0.9 c	0.66 ± 0.05 c
	2.00	15.0 ± 0.5 e	24.4 ± 0.4 c	26.9 ± 1.2 c	48.9 ± 1.5 d	1.10 ± 0.04 d
	0.50	19.2 ± 0.5 f	27.7 ± 0.8 d	41.5 ± 1.3 e	31.1 ± 2.1 f	1.50 ± 0.02 e
1.0	100	0 a	21.3 ± 0.4 ab	7.5 ± 0.3 a	71.2 ± 0.4 ab	0.35 ± 0.02 ab
	15.00	6.6 ± 0.4 b	22.7 ± 0.8 bc	11.8 ± 0.4 ab	65.7 ± 0.7 bc	0.52 ± 0.03 bc
	6.00	7.8 ± 0.1 bc	22.6 ± 0.7 bc	22.1 ± 1.4 c	55.4 ± 2.1 d	0.98 ± 0.03 d
	2.00	9.8 ± 0.4 bcd	23.7 ± 0.3 bc	35.4 ± 1.8 d	41.0 ± 2.1 e	1.49 ± 0.07 e
	0.50	12.3 ± 0.7 d	26.9 ± 0.6 d	59.4 ± 3.1 f	13.8 ± 3.6 g	2.21 ± 0.07 f

[a] Incubation medium contained 250 mM sucrose, 5 mM KH_2PO_4, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% BSA, 20 mM HEPES, 8 mM succinate, 0.15 mM Mb, and 0.5 or 1 mg MT protein/mL. [b] Values in columns with different letters are different ($p < 0.05$).

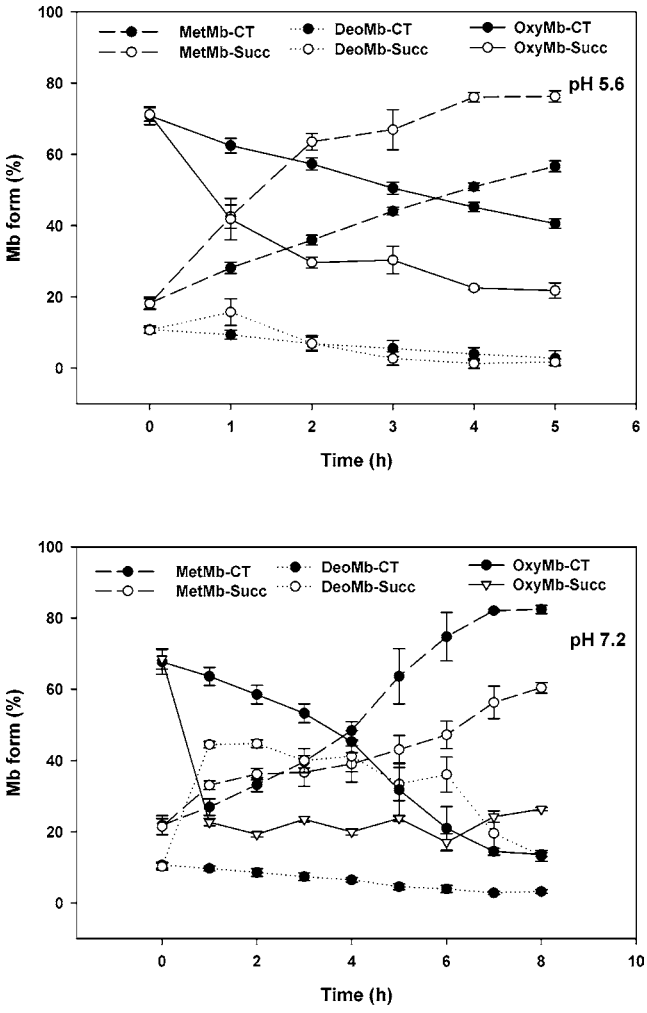

Figure 4. Changes in Mb form as affected by MT respiration over time in open systems at pH 5.6 and 7.2 and 25 °C. Succinate (Succ) treatments contained MT (1 mg MT protein/mL), Mb (0.15 mM), and succinate (8 mM). Controls (CT) lacked Succ. Standard error bars are indicated ($n = 3$).

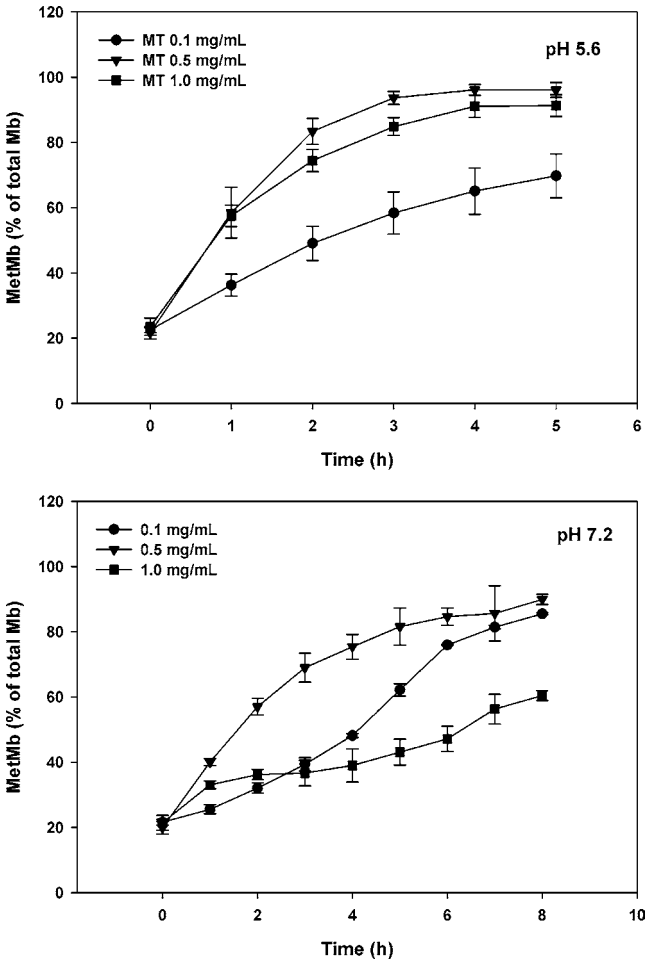

Figure 5. Effects of MT concentration on MetMb formation in open systems at pH 5.6 and 7.2 and 25 °C. MT (0.1, 0.5, or 1.0 mg MT protein/mL), Mb (0.15 mM), and Succ (8 mM) were included in systems. Standard error bars are indicated ($n = 3$).

As a result, more H_2O_2 was formed as a byproduct of this autoxidation at acidic pH; H_2O_2-induced DeoMb oxidation would be greater at acidic pH than at physiological pH. Therefore, our results suggested that DeoMb formation was favored by MT respiration at pH 5.6 and rapidly oxidized to MetMb, while greater stability to oxidation was achieved at pH 7.2. These results demonstrated that MT respiration facilitated formation of either DeoMb or MetMb in open systems in a pH-dependent manner.

Mitochondria are not evenly distributed in muscle fibers. Slow-contracting muscle fibers possess a greater MT concentration than do fast-contracting fibers (*41*). Therefore, the effects of MT density on MetMb formation were also evaluated in open systems. Mitochondria protein at a concentration of 0.5 mg/mL exhibited the greatest potential to increase MetMb formation at both pH values ($p < 0.05$; **Figure 5**). The MT density-dependent effect on MetMb formation at pH 5.6 may be attributed to different average pO_2 values inside the system where maximum Mb oxidation was observed at 1 to 1.4 mmHg pO_2 (*37*, *38*). At pH 7.2, MT respiration at a concentration of 1.0 mg/mL resulted in the lowest MetMb formation after 3 h

of incubation ($p < 0.05$; **Figure 5B**) and the responsible factor is unknown. Therefore, the effect of MT respiration on Mb redox status in open systems was pH- and MT density-dependent. The gaseous condition in open systems is similar to meat exposed to air, where pO_2 is determined by the rates of air diffusion into and oxygen consumption by the tissue (*10*). Our in vitro results provide direct evidence for MT and Mb interaction and support a basis for MT-mediated discoloration on the meat surface.

ABBREVIATIONS USED

MT, mitochondrial; Mb, myoglobin; OxyMb, oxymyoglobin; DeoMb, deoxymyoglobin; MetMb, metmyoglobin; DFD, dark, firm, and dry beef; pO_2, partial oxygen pressure; OCR, oxygen consumption rate; RCR, respiratory control ratio; ADP/O, the ratio ADP to oxygen consumption during MT phosphorylation; Tris-HCl, Tris[hydroxymethyl]aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; BSA, bovine albumin; EGTA, ethylene glycolbis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; MES, 2-[*N*-morpholino]ethanesulfonic acid; ADP, adenosine 5′-diphosphate.

ACKNOWLEDGMENT

We thank Dr. Stephen Daniels and Dr. Marie Cantino, Department of Physiology & Neurobiology, University of Connecticut, for their assistance with electron microscopy.

LITERATURE CITED

(1) Faustman, C.; Cassen, R. G. The biochemical basis for discoloration in fresh meat: A review. *J. Muscle Foods* **1990**, *1*, 217−243.

(2) Wittenberg, J. B. Myoglobin-facilitated oxygen diffusion: role of myoglobin in oxygen entry into muscle. *Physiol. Rev.* **1970**, *50*, 559−636.

(3) Wittenberg, B. A.; Wittenberg, J. B. Myoglobin-mediated oxygen delivery to mitochondria of isolated cardiac myocytes. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7503−7507.

(4) Wittenberg, B. A.; Wittenberg, J. B.; Caldwell, P. R. Role of myoglobin in the oxygen supply to red skeletal muscle. *J. Biol. Chem.* **1975**, *250*, 9038−9043.

(5) Gabbita, S. P.; Subramaniam, R.; Allouch, F.; Carney, J. M.; Butterfield, D. A. Effects of mitochondrial respiratory stimulation on membrane lipids and proteins: an electron paramagnetic resonance investigation. *Biochim. Biophys. Acta* **1998**, *1372*, 163−173.

(6) Cheah, K. S.; Cheah, A. M. Properties of mitochondria from ox neck muscle after storage *in situ*. *Int. J. Biochem.* **1974**, *5*, 753−760.

(7) Cheah, K. S.; Cheah, A. M. Post-mortem changes in structure and function of ox muscle mitochondria. 1. Electron microscopic and polarographic investigations. *J. Bioenerg.* **1971**, *2*, 85−92.

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

(9) Atkinson, J. L.; Follett, M. J. Biochemical studies on the discoloration of fresh meat. *J. Food Technol.* **1973**, *8*, 51−58.

(10) O'Keeffe, M.; Hood, D. E. Biochemical factors influencing metmyoglobin formation on beef from muscles of differing colour stability. *Meat Sci.* **1982**, *7*, 209−228.

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

(12) Madhavi, D. L.; Carpenter, C. E. Aging and processing affect color, metmyoglobin reductase and oxygen consumption of beef muscles. *J. Food Sci.* **1993**, *58*, 939−947.

(13) Cornforth, D. P.; Egbert, W. E.; Sisson, D. V. Effects of low temperature and increased oxygen concentration on color of intact pre-rigor muscle. *J. Food Sci.* **1985**, *50*, 1021−1024.

(14) Cornforth, D. P.; Egbert, W. R. Effect of rotenone and pH on the color of pre-rigor muscle. *J. Food Sci.* **1985**, *50*, 34−35, 44.

(15) Egbert, W. E.; Cornforth, D. P. Factors influencing color of dark cutting beef muscle. *J. Food Sci.* **1986**, *51*, 57−65.

(16) Brown, W. D.; Mebine, L. B. Autoxidation of oxymyoglobin. *J. Biol. Chem.* **1969**, *244*, 6696−6701.

(17) Smith, A. L. Preparation, properties, and conditions for assay of mitochondria: Slaughterhouse material, small-scale. *Methods Enzymol.* **1967**, *10*, 81−86.

(18) Estabrook, R. W. Mitochondrial respiratory control and the polarographic measurement of ADP: O ratios. *Methods Enzymol.* **1967**, *10*, 41−47.

(19) Bendall, J. R. Postmortem changes in muscle. In *The Structure and Function of Muscle*; Bourne, G. H., 2nd ed.; Academic Press: New York, 1973; pp 275−277.

(20) Schmedes, A.; Holmer, G. A new thiobarbituric acid (TBA) method for determining free malondialdehyde (MDA) and hydroperoxides selectively as a measure of lipid peroxidation. *JAOCS* **1989**, *66*, 813−817.

(21) Krzywicki, K. The determination of haem pigments in meat. *Meat Sci.* **1982**, *7*, 29−36.

(22) Tang, J.; Faustman, C.; Hoagland, T. A. Krzywicki revisited: Equations for spectrophotometric determination of myoglobin redox forms in aqueous meat extracts. *J. Food Sci.* **2004**, *69*, C717−720.

(23) SAS Institute Inc. *SAS User's Guide: Statistics*, 5th ed.; SAS Institute Inc.: Cary, NC, 1985.

(24) Tsvetkov, T.; Tsonev, L.; Meranzov, N.; Minkov, I. Functional changes in mitochondrial properties as a result of their membrane cryodestruction. II. Influence of freezing and thawing on ATP complex activity of intact liver mitochondria. *Cryobiology* **1985**, *22*, 111−118.

(25) Petrenko, A. A mechanism of latent cryoinjury and reparation of mitochondria. *Cryobiology* **1992**, *29*, 144−152.

(26) 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.* **1951**, *194*, 715−719.

(27) Bodwell, C. E.; Pearson, A. M.; Fennell, R. A. Post-mortem changes in muscle III. Histochemical observation in beef and pork. *J. Food Sci.* **1965**, *30*, 944−954.

(28) Ashmore, C. R.; Doerr, L.; Foster, G.; Carroll, F. Respiration of mitochondria isolated from dark-cutting beef. *J. Anim. Sci.* **1971**, *33*, 574−577.

(29) Ashmore, C. R.; Parker, W.; Doerr, L. Respiration of mitochondria isolated from dark-cutting beef: Postmortem changes. *J. Anim. Sci.* **1972**, *34*, 46−48.

(30) Cornforth, D. Color-its basis and importance. In *Quality Attributes and their Measurement in Meat, Poultry and Fish Products*, 1st ed.; Pearson, A. M., Dutson, T. R., Eds.; Blackie Academic & Professional: Glasgow, United Kingdom, 1994; pp 34−78.

(31) Takayanagi, R.; Takeshige, K.; Minakami, S. NADH- and NADPH-dependent lipid peroxidation in bovine heart submitochondrial particles. Dependence on the rate of electron flow in the respiratory chain and an antioxidant role of ubiquinol. *Biochem. J.* **1980**, *192*, 853−860.

(32) Meszaros, L.; Tihanyi, K.; Horvath, I. Mitochondrial substrate oxidation-dependent protection against lipid peroxidation. *Biochim. Biophys. Acta* **1982**, *713*, 675−677.

(33) Glinn, M.; Ernster, L.; Lee, C. P. Initiation of lipid peroxidation in submitochondrial particles: Effect of respiratory inhibitors. *Arch. Biochem. Biophys.* **1991**, *290*, 57−65.

(34) Lynch, M. P.; Faustman, C. Effect of aldehyde lipid oxidation products on myoglobin. *J. Agric. Food Chem.* **2000**, *48*, 600−604.

(35) Lynch, M. P.; Faustman, C.; Silbart, L. K.; Rood, D.; Furr, H. C. Detection of lipid-derived aldehydes and aldehyde: Protein adducts in vitro and in beef. *J. Food Sci.* **2001**, *66*, 1093−1099.

(36) Alderton, A. L.; Faustman, C.; Liebler, D. C.; Hill, D. W. Induction of redox instability of bovine myoglobin by adduction with 4-hydroxy-2-nonenal. *Biochemistry* **2003**, *42*, 4398−4405.
(37) George, P.; Stratmann, C. J. The oxidation of myoglobin to metmyoglobin by oxygen. 1. *Biochem. J.* **1952**, *51*, 103−108.
(38) George, P.; Stratmann, C. J. The oxidation of myoglobin to metmyoglobin by oxygen. 2. The relation between the first-order rate constant and the partial pressure of oxygen. *Biochem. J.* **1952**, *51*, 418−425.
(39) Wazawa, T.; Matsuoka, A.; Tajima, G.; Sugawara, Y.; Nakamura, K.; Shikama, K. Hydrogen peroxide plays a key role in the oxidation reaction of myoglobin by molecular oxygen. A computer simulation. *Biophys. J.* **1992**, *63*, 544−550.
(40) Shikama, K. M. A. Aplysia oxymyoglobin with an unusual stability property: Kinetic analysis of the pH dependence. *Biochemistry* **1986**, *25*, 3898−3903.
(41) McCormick, R. J. Structure and properties of tissues. In *Muscle Foods: Meat, Poultry and Seafood Technology*, 1st ed.; Kinsman, D. M., Kotula, A. W., Breidenstein, B. C., Eds.; Chapman & Hall: New York, 1994; pp 25−62.

Received for review August 11, 2004. Revised manuscript received November 15, 2004. Accepted November 24, 2004. This work was supported by the USDA-NRI, the Storrs Agricultural Experiment Station, University of Connecticut.

JF048646O