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Mitochondrial Reduction of Metmyoglobin: Dependence on the Electron Transport Chain

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Reduction of ferric myoglobin (metmyoglobin, MetMb) to its ferrous form is important for maintaining fresh meat color because only reduced myoglobin can bind oxygen to form the consumer-preferred cherry red color in fresh meat. The objective of this study was to characterize an apparent mitochondria electron transport chain (ETC)-linked pathway for MetMb reduction in vitro. MetMb was reduced in the presence of mitochondria and succinate ($p < 0.05$); mitochondria or succinate alone did not facilitate MetMb reduction relative to controls ($p > 0.05$). Flushing samples with oxygen greatly decreased MetMb reduction, while flushing with argon increased MetMb reduction when compared with controls ($p < 0.05$). ETC inhibitors were used to localize the site where electrons became available for MetMb reduction. MetMb reduction was increased by rotenone addition and decreased by malonic acid ($p < 0.05$); the reduction was completely abolished by additions of antimycin A or myxothiazol when compared with controls ($p < 0.05$). These results suggest that electrons become available for MetMb reduction at a site(s) between complex III and IV. Mitochondrial ETC-linked MetMb reduction increased with increased mitochondrial density and succinate concentration ($p < 0.05$); the greatest MetMb reduction was observed at pH 7.2 and 37 °C, and ETC-linked MetMb reducing activity decreased with time postmortem ($p < 0.05$). These results indicate that ETC-linked MetMb reduction exists but would be minimally active in postmortem muscles.

KEYWORDS: Metmyoglobin; mitochondria; electron-transport chain; reduction; succinate

INTRODUCTION

Myoglobin (Mb), an intracellular protein in skeletal and cardiac muscle, is closely associated with mitochondria in living cells. It serves as an oxygen reservoir and facilitates oxygen delivery to mitochondria, the major oxygen consuming organelle in cells (*1*−*4*). However, Mb is oxidized readily to physiologically inactive metmyoglobin (MetMb), especially at intracellular oxygen pressure (*1*, *5*−*7*). Mb is also the protein responsible for fresh meat color, an important sensory property affecting consumer acceptability (*8*, *9*). Meat discoloration from cherry red to brown is due to the accumulation of MetMb during storage. MetMb reduction mechanisms are critical for normal physiological functions of muscle and could be relevant to the maintenance of fresh meat color.

Brown et al. (*10*) and Koizumi et al. (*11*) reported that MetMb was reduced nonenzymatically by NADH or NADPH in the presence of EDTA or $MnCl_2$, and that the reduction was accelerated by the addition of H_2O_2 or various flavins, including FAD, flavin mononucleotide, and riboflavin (*10*, *11*). EDTA was proposed to inhibit metal-induced Mb autoxidation and permit detection of the reduction, while flavins played a role in mediating electron transfer (*10*, *11*).

Two fundamentally different enzymes have been reported to account for MetMb reduction, DT-diaphorase [NAD(P)H-quinone oxidoreductase], and MetMb reductase [NADH-cytochrome b_5 oxidoreductase] (*12*−*14*). DT-diaphorase catalyzes MetMb reduction in the presence of either NADH or NADPH; the majority (> 90%) of this enzyme was localized to the cytosol whereas minor activity was associated with mitochondrial and microsomal fractions (*14*−*16*). Bovine MetMb reductase was first purified and characterized from cardiac muscle by Hagler et al. (*13*). This enzyme is NADH-dependent and requires an artificial electron mediator such as methylene blue or ferrocyanide to reduce MetMb (*13*, *17*). The physiological mediators for MetMb reduction, cytochrome b_5 or OM cytochrome b_5 (a cytochrome b_5-like protein present in the outer mitochondrial membrane), have been isolated from rat liver and muscles (*18*−*21*). Arihara and fellow workers (*22*) further characterized MetMb reductase in subcellular fractions from bovine muscle. MetMb reductase was found predominantly in the mitochondrial fraction but was also detected at lower levels in the microsomal

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fraction; OM cytochrome b_5 was located primarily in the mitochondrial fraction, whereas cytochrome b_5 was detected only in the microsomal fraction (*22*). These results demonstrated that mitochondria and microsomes possess complete MetMb reduction enzyme systems capable of mediating MetMb reduction.

The involvement of mitochondria in MetMb reduction was proposed by Watts et al. (*23*). They reported that the addition of NAD^+ to ground beef generated NADH and therefore increased MetMb reduction; this reduction was inhibited by the addition of amytal or rotenone, two electron-transfer chain complex I inhibitors. Anaerobic conditions (N_2) also increased this NAD^+-mediated MetMb reduction. Thus, they concluded that the role of mitochondria in the observed reduction was to establish anaerobic conditions to facilitate MetMb reduction by transferring electrons from NADH or succinate to oxygen. They did not consider the possibility that MetMb was reduced directly by the mitochondrial electron transport chain (ETC); anaerobiosis is necessary for this reduction. In the present study, we sought to characterize a mitochondria ETC-linked pathway for MetMb reduction. Since succinate oxidation does not involve the generation of NADH, it would be an ideal substrate used for this study.

MATERIALS AND METHODS

Materials and Chemicals. Beef hearts were obtained locally within 1 h of exsanguination, placed on ice, and transported to the laboratory. Samples for 96 h, 192 h, and 45 days were vacuum-packaged and stored at 4 °C. Equine heart Mb, $MgCl_2$, bovine serum albumin (BSA, essentially fatty acid free), sucrose, tris[hydroxymethyl]aminomethane hydrochloride (Tris-HCl), ethylenediaminetetraacetic acid (EDTA), 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, rotenone, myxothiazol, antimycin A, malonic acid, and Nagarse protease (10.5 units/mg) were obtained from Sigma Chemical Co. (St. Louis, MO); rotenone, myxothiazol, and antimycin A were dissolved in ethanol, and control reactions contained an equivalent volume of ethanol only. Sodium succinate was purchased from Fisher Scientific (Fair Lawn, NJ). All chemicals were reagent grade.

MetMb Preparation. Commercial equine heart Mb was dissolved in 50 mM Tris-HCl buffer (pH 7.4), and the MetMb concentration was determined to be 96% by a spectrophotometric method (*24*).

Bovine Mitochondria Isolation. Except for the experiment regarding the effect of time postmortem (2, 96, 192 h, and 45 days) on MetMb reduction, mitochondria were isolated at 2 h postmortem from bovine cardiac muscle according to Smith (*25*) with minor modification. Briefly, 100 g of ground cardiac muscle was washed with 250 mM sucrose twice and suspended in 200 mL of mitochondrial isolation buffer (250 mM sucrose, 10 mM Tris-HCl, 1 mM EGTA, 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 1 L with mitochondrial 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 one pass with a Wheaton Potter-Elvehjem grinder (Millville, NJ); 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 (26 000*g*) for 15 min. The pellet was washed twice, suspended in mitochondrial suspension buffer (250 mM sucrose, 10 mM Tris-HCl, pH 7.4), and then used. All steps were performed at 0−4 °C. Mitochondrial protein content was determined by a Bicinchoninic Acid Protein Assay Kit from Sigma Chemical Co. (St. Louis, MO).

Mitochondrial Oxygen Consumption. Mitochondrial 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 temperature was maintained at 37 °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, Great Britain) 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, 20 mM HEPES). Calculations for state IV oxygen consumption rate (OCR) were based on the methods of Estabrook (*26*).

Reduction of MetMb. MetMb (2.5 mg/mL) reduction was conducted in a microfuge open top tube at pH 7.2 (120 mM KCl, 5 mM KH_2PO_4, 30 mM Tris-HCl), 6.4 (120 mM KCl, 5 mM KH_2PO_4, 30 mM MES), or 5.6 (120 mM KCl, 5 mM KH_2PO_4, 30 mM maleic acid) and 37, 25, or 4 °C. At specific time points, samples were removed and centrifuged (15 000*g*) with an Eppendorf 5415D centrifuge (Westbury, NY) for 3 min. The resulting supernatant was scanned from 650 to 500 nm with a Shimadzu UV-2101PC spectrophotometer (Kyoto, Japan). The relative proportions of deoxymyoglobin (DeoMb), oxymyoglobin (OxyMb), and MetMb were calculated according to Tang et al. (*24*). Mitochondrial ETC inhibitors including rotenone, malonic acid, anitmycin A, and myxothiazol were used in reduction systems; control experiments did not show any effects of these inhibitors on OxyMb oxidation or MetMb reduction (results not shown). Lipid oxidation was also monitored as thiobarbituric reactive substances (*27*) in our reaction systems, as products of this breakdown process have been shown to bind to OxyMb and increase MetMb formation (*28*−*30*); however, no lipid oxidation was observed in our reaction systems (results not shown).

Gas Flushing Treatments. Samples (mitochondria 1 mg/mL, MetMb 2.5 mg/mL, 8 mM succinate, pH 7.2) were prepared in Warburg flasks for ease of gas flushing and then incubated at 37 °C and flushed continuously with ultrahigh purity argon or 80% oxygen (balanced with N_2). Controls were not flushed. Samples were removed from Warburg flasks after 2 h flushing, centrifuged (15 000*g*) for 3 min, and then scanned from 650 to 500 nm with a Shimadzu UV-2101PC spectrophotometer.

Statistical Analysis. Results were expressed as mean values of three independent trials. Data were analyzed using the GLM procedure of SAS, and differences among means were detected at the 5% level using LSD with appropriate correction for multiple comparisons (*31*).

RESULTS

Mitochondrial Electron Transport Chain-Linked Reduction of MetMb. MetMb was incubated with mitochondria and/or succinate at pH 7.2 and 37 °C for 3 h. During incubation, an absorbance increase at 544 and 582 nm was observed and indicated that MetMb was reduced in the presence of succinate and mitochondria ($p < 0.05$; **Figure 1**); mitochondria or succinate alone did not facilitate MetMb reduction relative to controls ($p > 0.05$; **Figure 1**). These results demonstrated that both succinate and mitochondria were required for MetMb reduction.

To further characterize ETC involvement in MetMb reduction and localize MetMb reduction to a specific site of the ETC, rotenone, malonic acid, antimycin A, and myxothiazol were used. In general, there was an inhibitor-specific effect on MetMb reduction when compared with controls ($p < 0.05$; **Figure 2**), and this supported involvement of the ETC in the observed MetMb reduction. Rotenone addition led to increased mitochondrial oxygen consumption and increased MetMb reduction relative to controls ($p < 0.05$; **Figure 2**). Malonic acid, a complex II inhibitor, decreased mitochondrial oxygen consumption rate (OCR) to 70.6% of controls and also decreased MetMb

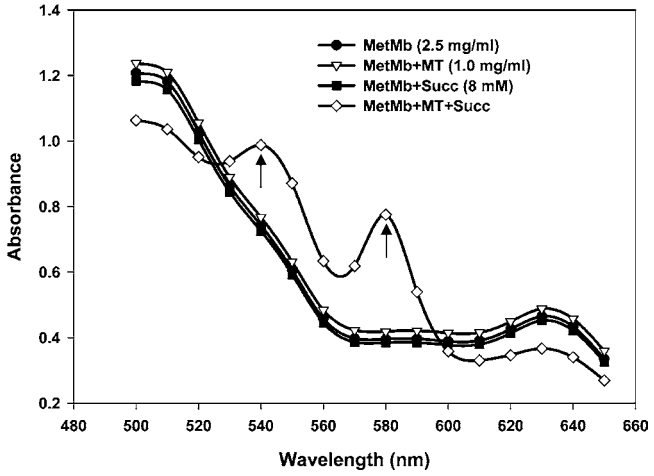

Figure 1. The effect of succinate (Succ, 8 mM) on metmyoglobin (MetMb, 2.5 mg/mL) reduction in the presence or absence of mitochondria (MT, 1 mg/mL) following 3 h at pH 7.2 and 37 °C. Absorbance increases at 544 and 582 nm (depicted by arrows) indicates MetMb reduction.

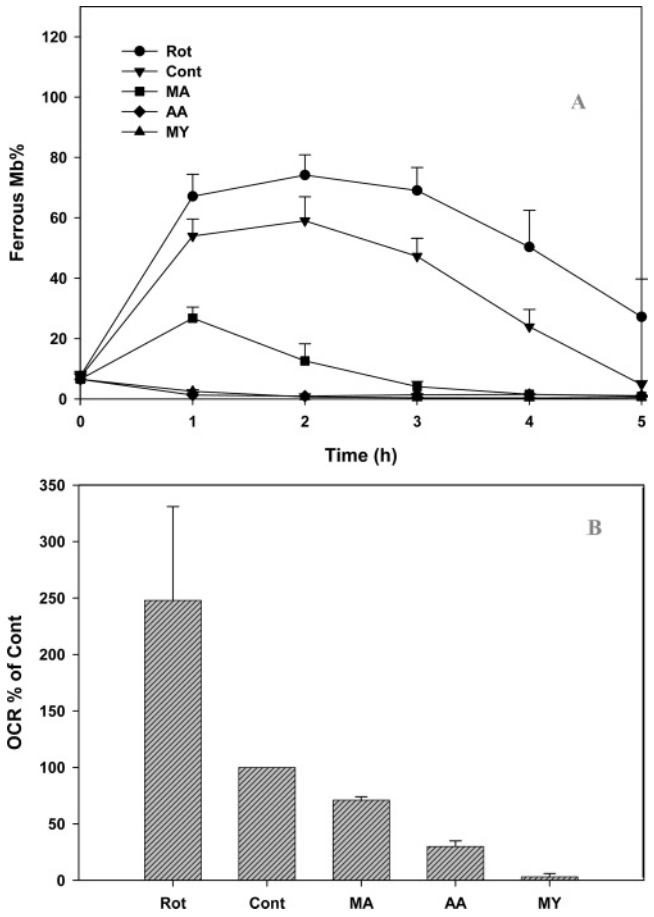

Figure 2. The effect of inhibitors on metmyoglobin (MetMb, 2.5 mg/mL) reduction (**A**), and mitochondrial (MT) state IV oxygen consumption rate (OCR) (**B**), in the presence of MT (1.0 mg/mL) and succinate (8 mM) at pH 7.2 and 37 °C. The inhibitors were rotenone (Rot, 0.02 mM), malonic acid (MA, 10 mM), antimycin A (AA, 0.01 mM), and myxothiazol (MY, 0.01 mM). MT state IV OCR expressed as a % of the control (Cont). Standard error bars are indicated.

reduction relative to controls ($p < 0.05$; **Figure 2**). Complex III inhibitors, antimycin A and myxothiazol, inhibited 69.6% and 96.7% of oxygen consumption relative to controls, respectively, and completely abolished MetMb reduction ($p < 0.05$; **Figure 2**). These results suggested that electrons became

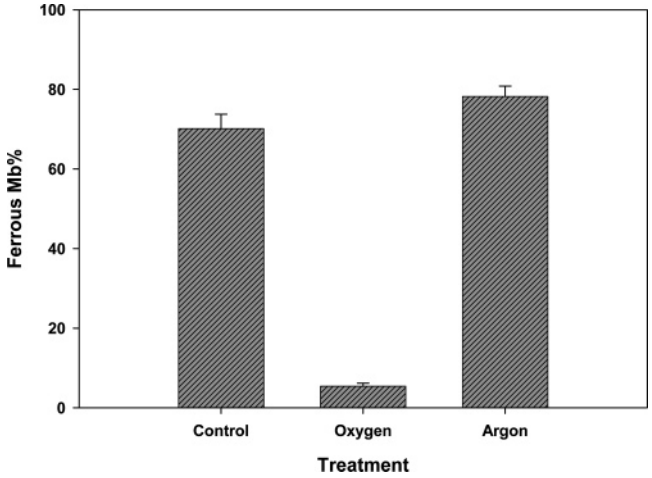

Figure 3. The effect of oxygen or argon on metmyoglobin (MetMb) reduction in the presence of mitochondria (MT) and succinate at pH 7.2 and 37 °C for 2 h. Each system contained 120 mM KCl, 30 mM Tris-HCl, 5 mM KH_2PO_4, 8 mM succinate, 1.0 mg MT protein/ml, and 2.5 mg/mL MetMb.

available for ETC-linked MetMb reduction at site(s) beyond the Q cycle of complex III, as myxothiazol inhibits electron transfer from ubiquinol of Q cycle to Rieske Fe-S protein (*32*).

Inhibitors for complex IV include compounds such as cyanide, azide, and CO, and that also combine with heme iron to alter heme protein absorbance spectra; this negates their usefulness for experiments involving Mb (*23*). As an alternative approach, reaction systems were flushed with argon or oxygen to explore the location of possible reduction site(s), as oxygen is the final electron acceptor and anaerobiosis results in electrons accumulating inside the ETC. It is also important to note that high oxygen partial pressure (pO_2) decreases the rate of Mb oxidation (*7*, *33*). Flushing samples with argon increased MetMb reduction, while oxygen inclusion decreased MetMb reduction ($p < 0.05$; **Figure 3**). These results suggested that lower pO_2 or anaerobic conditions were required for ETC dependent reduction of MetMb in this system.

ETC-Linked MetMb Reduction Affected by pH, Temperature, Mitochondrial or Succinate Concentrations, and Age Postmortem. ETC-linked MetMb reduction was greater at increased pH (pH 5.6 < 6.4 < 7.2) and temperature (4 < 25 < 37 °C), with the greatest MetMb reduction observed at pH 7.2 and 37 °C ($p < 0.05$; **Figure 4**). This is not surprising because these conditions generally represent physiological pH and temperature for mammals. Lower pH also enhances OxyMb oxidation (*34*), and it is likely that decreased MetMb reducing activity and increased Mb oxidation both contributed to the measured decrease in MetMb reduction at lower pH values.

Increasing mitochondrial density above 0.1 mg mitochondrial protein/mL assay volume increased MetMb reduction ($p < 0.05$; **Figure 5A**); there was no difference between controls and the 0.1 mg mitochondrial protein/ml treatment ($p > 0.05$; **Figure 5A**). Similarly, increasing succinate concentrations led to greater MetMb reduction as demonstrated by extended reduction times ($p < 0.05$; **Figure 5B**). MetMb reduction reached a maximum at 4 h in the presence of 8 mM succinate, and the reduced Mb was completely reoxidized again at 10 h. Increasing succinate concentrations above 8 mM did not increase the maximum concentration of reduced Mb but extended the reduction period when compared with 8 mM succinate. This result suggested that succinate was required to maintain Mb in the reduced form and that when succinate was exhausted, Mb autoxidation exceeded MetMb reduction.

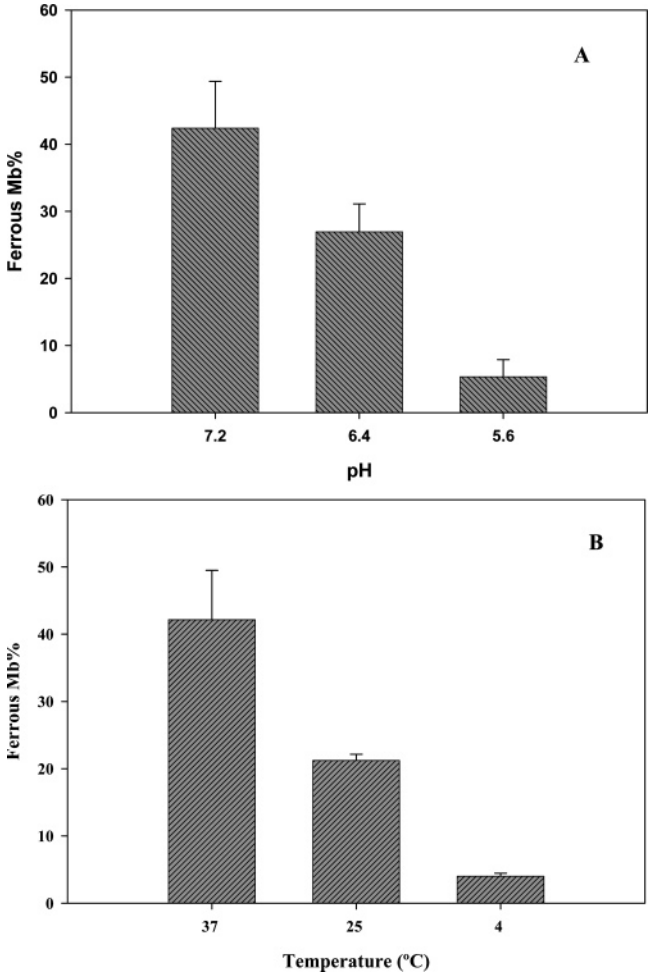

Figure 4. The effect of pH (**A**) or temperature (**B**) on ETC-linked metmyoglobin (MetMb, 2.5 mg/mL) reduction. Samples were incubated at 37 °C (**A**) or pH 7.2 (**B**) for 3 h. Standard error bars are indicated.

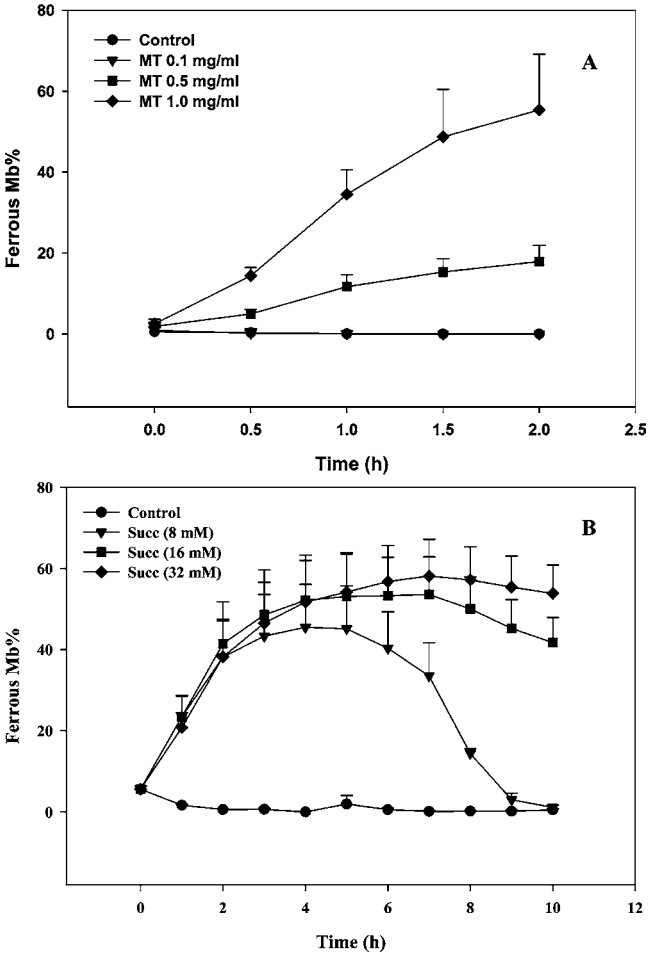

Figure 5. The effect of mitochondrial (MT, **A**) or succinate (Succ, **B**) concentrations on ETC-linked metmyoglobin (MetMb, 2.5 mg/mL) reduction at pH 7.2 and 37 °C. For **A**, the succinate concentration was 8 mM; for **B**, the mitochondria were used as 1 mg mitochondrial protein per ml. Standard error bars are indicated.

Mitochondria were isolated at 2 h, 96 h, 192 h, and 45 days postmortem. ETC-linked MetMb reduction decreased with time postmortem; MetMb reduction was greatest with mitochondria isolated at 2 h postmortem ($p < 0.05$; **Figure 6**). There was no significant difference in MetMb reduction between mitochondria isolated at 96 h and at 192 h ($p > 0.05$; **Figure 6**). Mitochondria isolated at 45 days showed the least ETC-linked MetMb reducing activity ($p < 0.05$). Mitochondrial integrity is lost with age postmortem (*35*), and this may explain the decreased MetMb reduction and rapid discoloration of retail meat from vacuum-aged primals (*36*, *37*).

DISSCUSSION

The oxidation of succinate was previously reported to enhance MetMb reduction in ground beef (*23*); however, the role of succinate in this reaction was proposed to increase mitochondrial respiration and enhance the establishment of anaerobic conditions that subsequently facilitated enzymatic reduction of MetMb (*23*). Our results in vitro demonstrated that succinate addition in the presence of mitochondria led to significant reduction of MetMb, and this reduction was affected by ETC inhibitors and oxygen (**Figures 1**, **2**, and **3**). MetMb reduction was not observed with mitochondria flushed with argon in the absence of succinate (results not shown). Therefore, it is unlikely that succinate oxidation acted solely by decreasing pO_2 to facilitate enzymatic MetMb reduction in our experiments. A reasonable explanation for the observed results is that MetMb was reduced

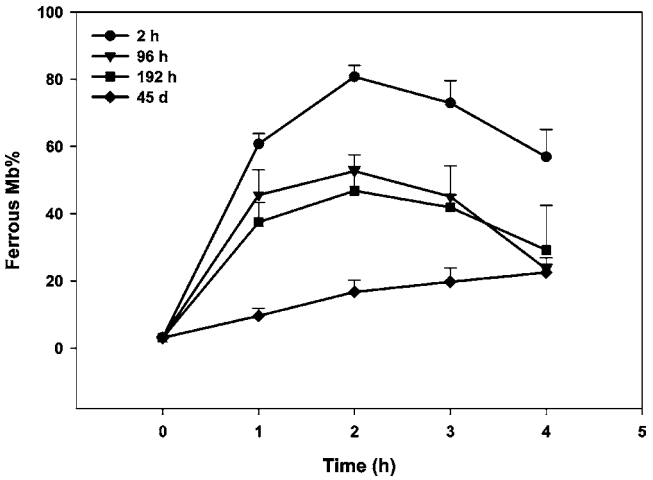

Figure 6. The effects of time postmortem on ETC-linked metmyoglobin (MetMb, 2.5 mg/mL) reduction in the presence of succinate (8 mM) and mitochondria (1 mg/mL) at pH 7.2 and 37 °C. Standard error bars are indicated.

by a mitochondrial ETC-linked pathway, and this reduction occurred only under lower pO_2 and/or when electrons accumulated inside the ETC. In our reaction systems, lower pO_2 was established by mitochondrial respiration that resulted in oxygen concentrations decreasing to 0% within 5 min in closed

systems containing 1 mg mitochondrial protein/mL and 8 mM succinate (results not shown). This pathway also provided insight relative to the effect of oxygen on MetMb reduction. Early on, Watts et al. (*23*) and Walters et al. (*38*) reported that lower pO_2 or anaerobiosis facilitated or increased MetMb reduction. However, neither enzymatic or nonenzymatic pathways require lower pO_2 for MetMb reduction and appears to increase MetMb reduction under such conditions (*10*, *11*, *13*, *14*). The observations by Watts et al. (*23*) and Walters et al. (*38*) were obtained with postmortem muscle in which mitochondria were present and likely functional. Therefore, it is possible that the mitochondrial ETC-linked pathway was responsible for their observation that MetMb reduction increased under lower pO_2 or in the absence of oxygen.

Succinate oxidation in the Krebs cycle does not produce NADH, and electrons from succinate oxidation are delivered directly to ubiquinone via succinate dehydrogenase (**Figure 7**). Normally, electrons move to complexes III and IV and ultimately reduce oxygen; however, they can also move in a reverse direction to complex I if oxygen is absent or if complexes III or IV are inhibited (*39*, *40*). Giddings (*41*) proposed that reversed electron-transfer had the potential to produce NADH by NADH dehydrogenase in complex I, thereby facilitating reduction of MetMb. In our experiment, no NAD^+ was added and rotenone (complex I inhibitor) addition still increased ETC-dependent MetMb reduction (**Figure 2**). These results eliminated the possibility that reversed electron transfer was responsible for MetMb reduction by succinate and mitochondria. Rather, the increase in MetMb reduction by rotenone addition was attributed to increased electron flux to ETC complex IV, presumably because rotenone blocked reversed electron transport from ubiquinol to complex I (*42*). Watts et al. (*23*) and Govindarajan et al. (*43*) observed increased discoloration in ground beef upon the addition of rotenone, an inhibitor of electron transfer between complex I and coenzyme Q of the ETC. In their experiments, ground beef was used without added substrates. Thus, NADH produced by muscle could be used as substrate for MetMb reduction, and MetMb reduction could be inhibited by rotenone addition, if the ETC was the primary reduction pathway in these investigators' experiments.

The effects of specific inhibitors helped elucidate the site(s) at which electrons could become available for MetMb reduction (**Figure 7**). For ETC-dependent reduction of MetMb by succinate (**Figures 2** and **7**), electron flow through complexes II and III was clearly required as MetMb reduction was prevented by the addition of the complex II inhibitor malonic acid and complex III inhibitors antimycin A and myxothiazol. It was reported previously that blockage of respiratory ETC with antimycin A initiated superoxide anion production (*32*, *42*, *44*) which can potentially suppress MetMb reduction (*45*−*47*). However, myxothizaol, which blocks the oxidation of ubiquinol before an electron can be donated to the Rieske Fe-S protein, does not generate appreciable superoxide in intact mitochondria (*44*). Our observation that myxothiazol also blocked succinate-dependent MetMb reduction suggests that electron movement through the Q cycle is required for the effect (**Figures 2** and **7**). Flushing with argon increased ETC-dependent MetMb reduction, while oxygen completely abolished it. Oxygen is the final electron acceptor in the mitochondrial ETC, and its exclusion would lead to accumulation of electrons. This would increase reducing potential thereby providing a pool of electrons available for MetMb reduction (*48*). Our results appear to suggest that electrons were delivered for MetMb reduction from

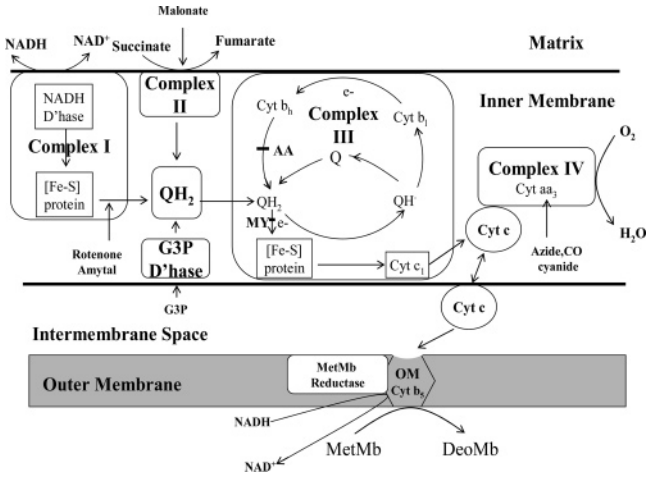

Figure 7. Potential pathways for MetMb reduction mediated by the mitochondrial electron transport chain (ETC). Rotenone, amytal, malonate, antimycin A, myxothiazol, azide, CO, and cyanide are mitochondrial ETC inhibitors and their action sites noted. Abbreviations used: cyt aa_3, cytochrome a/a_3; AA, antimycin A; cyt b_h, cytochrome b with heme h (cytochrome b_{562}); cyt b_l, cytochrome b with heme l (cytochrome b_{566}); cyt c, cytochrome *c*; cyt c_1, cytochrome c_1; G3P, glycerol 3-phosphate; G3P D′hase, glycerol 3-phosphate dehydrogenase; [Fe-S] protein; iron–sulfur protein; MY, myxothiazol; NADH D′hase, NADH dehydrogenase; Q, ubiquinone; QH·, ubisemiquinone radical; QH_2, ubiquinol; OM cyt b_5, outer membrane cytochrome b_5; MetMb reductase, NADH-cytochrome b_5 reductase.

a site(s) between the Rieske Fe-S protein of complex III and heme a_3-Cu_B center of complex IV (**Figure 7**) (*48*).

Our results suggest that electrons from the ETC by succinate oxidation were responsible for MetMb reduction. Unlike triphenyltetrazolium chloride (*49*), α-tocopherol (*50*), and ascorbic acid (*44*), all of which have been reduced by the mitochondrial ETC, Mb is a sarcoplasmic protein and too large to cross the mitochondrial outer membrane. Thus, an electron-transfer mediator would be needed to move electrons from the ETC to MetMb. Cytochrome *c* is a protein that functions to transfer electrons from mitochondrial complexes III to IV. Some cytochrome *c* molecules are located in the inner mitochondrial membrane, while others are associated through electrostatic interactions with the outer surface of the mitochondrial inner membrane and can be released into the intermembrane space (*48*, *51*, *52*). Cytochrome *c* has been suggested to be an electron shuttle between the outer and inner mitochondrial membrane during aerobic oxidation of cytosolic NADH (*53*). This pathway is insensitive to rotenone, antimycin A, or myxothiaxol and involved in the pathway (*53*−*56*): NADH → NADH-cytochrome b_5 reductase → OM cytochrome b_5 → intermembrane cytochrome *c* → cytochrome *c* oxidase → oxygen. It is interesting to note that some components of this pathway have been suggested to be involved in enzymatic reduction of MetMb by NADH (*19*, *22*). Therefore, we propose that the ETC-linked reduction of MetMb with succinate that we observed could follow the pathway: succinate → complex II → ubiquinone → complex III → cytochrome *c* → OM cytochrome b_5 → MetMb (**Figure 7**). The formation of a cytochrome b_5:cytochrome *c* complex to facilitate electron transfer has been reported in vitro (*57*−*59*). Further studies are necessary to ascertain the existence of this pathway for MetMb reduction in vivo.

Meat is different from living muscle, and the conversion of muscle to meat is complex (*60*). Muscle pH decreases from the point of death to rigor mortis and displays a general ultimate pH of 5.6 for normal meat and approximately pH 6.4 for dark,

firm, and dry meat. Temperatures of storage are typically at 4 °C in coolers. ETC-linked MetMb reduction in vitro was greatly diminished at pH values and temperatures associated with meat (**Figure 4**). Tang et al. (*35*) reported that the effect of succinate-dependent mitochondrial respiration on OxyMb oxidation was dependent on pH; mitochondrial respiration enhanced OxyMb oxidation to MetMb at pH 5.6 while it increased conversion of OxyMb to DeoMb at pH 7.2 (*35*). Based on present results, active MetMb reduction by an ETC-linked pathway was likely to occur at pH 7.2 and may be partly responsible for greater DeoMb and less MetMb formation observed previously.

ETC-linked MetMb reduction was increased with increased concentrations of succinate or mitochondria (**Figure 5**). Most relevant substrates are in low concentrations in postmortem muscle even though most enzyme systems are still active (*61*, *62*). Thus, substrate availability may be another factor that could limit ETC-linked MetMb reduction in postmortem muscle. Tang et al. previously investigated the effect of mitochondrial density on OxyMb oxidation; MetMb formation was least at a mitochondrial protein concentration of 1.0 mg/mL, followed by 0.1 mg/mL and 0.5 mg/mL at pH 7.2 (*35*). Our present results demonstrated that the greatest MetMb reduction occurred at a mitochondrial concentration of 1.0 mg/mL (**Figure 5**). Taken together, these results suggest that the minimal MetMb formation observed previously (*35*) resulted from active MetMb reduction by an ETC-linked pathway.

An ETC-linked pathway for MetMb reduction was identified. The possible site(s) where electrons became available for MetMb reduction appears to be located between complexes III and IV. ETC-linked MetMb reduction was decreased with decreased pH, lower temperature, and time postmortem; it was increased with increased mitochondrial density and succinate concentration.

ABBREVIATIONS USED

Mb, myoglobin; OxyMb, oxymyoglobin; DeoMb, deoxymyoglobin; MetMb, metmyoglobin; 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; OCR, oxygen consumption rate; ETC, electron transport chain; pO_2, partial oxygen pressure.

LITERATURE CITED

(1) Wittenberg, J. B.; Wittenberg, B. A. Myoglobin function reassessed. *J. Exp. Biol.* **2003**, *206*, 2011−2020.

(2) 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.

(3) 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.

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

(5) Wittenberg, B. A.; Wittenberg, J. B. Oxygen pressure gradients in isolated cardiac myocytes. *J. Biol.* Chem. **1985**, *260*, 6548−6554.

(6) 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.

(7) 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.

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

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

(10) 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.

(11) Koizumi, C.; Brown, W. D. A peroxidative mechanism for the nonenzymatic reduction of metmyoglobin. *Biochim. Biophys. Acta* **1972**, *264*, 17−24.

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

(13) Hagler, L.; Coppes, R. I., Jr.; 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.

(14) Lind, C.; Cadenas, E.; Hochstein, P.; Ernster, L. DT-diaphorase: purification, properties, and function. *Methods Enzymol.* **1990**, *186*, 287−301.

(15) Taylor, D.; Hochstein, P. Reduction of metmyoglobin in myocytes. *J. Mol. Cell Cardiol.* **1982**, *14*, 133−140.

(16) Conover, T. E.; Danielson, L.; Ernster, L. DT diaphorase: III. Separation of mitochondrial DT diaphorase and respiratory chain. *Biochim. Biophys. Acta* **1963**, *67*, 254−267.

(17) Arihara, K.; Itoh, M.; Kondo, Y. Identification of bovine skeletal muscle metmyoglobin reductase as a NADH-cytochrome b5 reductase. *Jpn. J. Zootech. Sci.* **1989**, *60*, 46−56.

(18) Arihara, K.; Itoh, M.; Kondo, Y. Detection of cytochrome b5 in bovine skeletal muscle by electrophoretic immunoblotting technique. *Jpn. J. Zootech. Sci.* **1989**, *60*, 97−100.

(19) Arihara, K.; Indo, M.; Itoh, M.; Kondo, Y. Presence of cytochrome b5-like hemoprotein (OM cytochrome b) in rat muscles as a metmyoglobin reducing enzyme system component. *Jpn. J. Zootech. Sci.* **1990**, *61*, 837−842.

(20) Ito, A. Cytochrome b5-like hemoprotein of outer mitochondrial membrane: OM cytochrome b. II. contribution of OM cytochrome b to rotenone-insensitive NADH-cytochrome *c* reductase activity. *J. Biochem. (Tokyo)* **1980**, *87*, 73−80.

(21) Ito, A. Cytochrome b5-like hemoprotein of outer mitochondrial membrane: OM cytochrome b. I. purification of OM cytochrome b from rat liver mitochondria and comparison of its molecular properties with those of cytochrome b5. *J. Biochem. (Tokyo)* **1980**, *87*, 63−71.

(22) Arihara, K.; Cassens, R. G.; Greaser, M. L.; Luchansky, J. B.; Mozdiak, P. E. Localization of metmyoglobin-reducing enzyme (NADH-cytochrome b5 reductase) system components in bovine skeletal muscle. *Meat Sci.* **1995**, *39*, 205−213.

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

(24) 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.

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

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

(27) Schmedes, A., and 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.
(28) Lynch, M. P.; Faustman, C. Effect of aldehyde lipid oxidation products on myoglobin. *J. Agric. Food* Chem. **2000**, *48*, 600−604.
(29) 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.
(30) 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.
(31) SAS Institute Inc. *SAS User's Guide: Statistics*, 5 ed.; SAS Institute Inc.: Cary, NC, 1985.
(32) Turrens, J. F. Superoxide production by the mitochondrial respiratory chain. *Biosci. Rep.* **1997**, *17*, 3−8.
(33) George, P.; Stratmann, C. J. The oxidation of myoglobin to metmyoglobin by oxygen: 1. *Biochem. J.* **1952**, *51*, 103−108.
(34) Yin, M. C.; Faustman, C. Influence of temperature, pH, and phospholipid composition upon the stability of myoglobin and phospholipid: a liposome model. *J. Agric. Food Chem.* **1993**, *41*, 853−857.
(35) Tang, J.; Faustman, C.; Hoagland, T. A. Postmortem oxygen consumption of mitochondria and its effects on myoglobin form and stability. *J. Agric. Food Chem.* **2005**, *53*, 1223−1230.
(36) Bevilacqua, A. E.; Zaritzky, N. E. Rate of pigment modifications in packaged refrigerated beef using reflectance spectrophotometry. *J. Food Process. Preserv.* **1986**, *10*, 1−18.
(37) Hood, D. E. Factors affecting the rate of metmyoglobin accumulation in prepackaged beef. *Meat Sci*. **1980**, *4*, 247−265.
(38) Walters, B. M.; Taylor, M. M. M. Biochemical properties of pork muscle in relation to curing. *Food Technol*. **1963**, *17*, 354.
(39) Low, H.; Krueger, H.; Ziegler, D. M. On the reduction of externally added DPN by succinate in submitochondrial particles. *Biochem. Biophys. Res. Commun.* **1961**, *5*, 231−237.
(40) Low, H.; Vallin, I. Reduction of added DPN from the cytochrome *c* level in submitochondrial particles. *Biochem. Biophys. Res. Commun.* **1962**, *9*, 307−312.
(41) Giddings, G. G. Reduction of ferrimyoglobin in meat. *CRC Crit. Rev. Food Technol.* **1974**, *5*, 143−173.
(42) Liu, Y.; Fiskum, G.; Schubert, D. Generation of reactive oxygen species by the mitochondrial electron transport chain. *J. Neurochem.* **2002**, *80*, 780−787.
(43) Govindarajan, S.; Hultin, H. O.; Kotula, A. W. Myoglobin oxidation in ground beef: mechanistic studies. *J Food Sci*. **1977**, *42*, 571−577, 582.
(44) Li, X.; Cobb, C. E.; May, J. M. Mitochondrial recycling of ascorbic acid from dehydroascorbic acid: dependence on the electron transport chain. *Arch. Biochem. Biophys.* **2002**, *403*, 103−110.
(45) Radi, R.; Turrens, J. F.; Chang, L. Y.; Bush, K. M.; Crapo, J. D.; Freeman, B. A. Detection of catalase in rat heart mitochondria. *J. Biol. Chem.* **1991**, *266*, 22028−22034.
(46) Tang, J.; Faustman, C.; Lee, S.; Hoagland, T. A. Effect of glutathione on oxymyoglobin oxidation. *J. Agric. Food Chem.* **2003**, *51*, 1691−1695.
(47) Yusa, K.; Shikama, K. Oxidation of oxymyoglobin to metmyoglobin with hydrogen peroxide: involvement of ferryl intermediate. *Biochemistry* **1987**, *26*, 6684−6688.
(48) Nelson, D. L.; Cox, M. M. *Lehninger Principles of Biochemistry*, 3rd ed.; Worth Publishers: New York, 2000.
(49) Rich, P. R.; Mischis, L. A.; Purton, S.; Wiskich, J. T. The sites of interaction of triphenyltetrazolium chloride with mitochondrial respiratory chains. *FEMS Microbiol. Lett.* **2001**, *202*, 181−187.
(50) Maguire, J. J.; Wilson, D. S.; Packer, L. Mitochondrial electron transport-linked tocopheroxyl radical reduction. *J. Biol. Chem.* **1989**, *264*, 21462−21465.
(51) Cortese, J. D.; Voglino, A. L.; Hackenbrock, C. R. Persistence of cytochrome *c* binding to membranes at physiological mitochondrial intermembrane space ionic strength. *Biochim. Biophys. Acta* **1995**, *1228*, 216−228.
(52) Ott, M.; Robertson, J. D.; Gogvadze, V.; Zhivotovsky, B.; Orrenius, S. Cytochrome *c* release from mitochondria proceeds by a two-step process. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 1259−1263.
(53) Bernardi, P.; Azzone, G. F. Cytochrome *c* as an electron shuttle between the outer and inner mitochondrial membranes. *J. Biol. Chem.* **1981**, *256*, 7187−7192.
(54) Bodrova, M. E.; Dedukhova, V. I.; Mokhova, E. N.; Skulachev, V. P. Membrane potential generation coupled to oxidation of external NADH in liver mitochondria. *FEBS Lett.* **1998**, *435*, 269−274.
(55) Lemeshko, V. V. Mg(2+) induces intermembrane electron transport by cytochrome *c* desorption in mitochondria with the ruptured outer membrane. *FEBS Lett.* **2000**, *472*, 5−8.
(56) Sottocasa, G. L.; Kuylenstierna, B.; Ernster, L.; Bergstrand, A. An electron-transport system associated with the outer membrane of liver mitochondria. A biochemical and morphological study. *J. Cell Biol.* **1967**, *32*, 415−438.
(57) Mauk, M. R.; Reid, L. S.; Mauk, A. G. Spectrophotometric analysis of the interaction between cytochrome b5 and cytochrome *c*. *Biochemistry* **1982**, *21*, 1843−1846.
(58) Shao, W.; Im, S. C.; Zuiderweg, E. R.; Waskell, L. Mapping the binding interface of the cytochrome b5-cytochrome *c* complex by nuclear magnetic resonance. *Biochemistry* **2003**, *42*, 14774−14784.
(59) Banci, L.; Bertini, I.; Felli, I. C.; Krippahl, L.; Kubicek, K.; Moura, J. J.; Rosato, A. A further investigation of the cytochrome b5-cytochrome *c* complex. *J. Biol. Inorg. Chem.* **2003**, *8*, 777−786.
(60) Greaser, M. L. Conversion of muscle to meat. *In Muscle As Food* **1986**, 37−102.
(61) Andrews, M. M., Guthneck, B. T., McBride, B. H., and Schweigert, B. S. Stability of certain respiratory and glycolytic enzyme systems in animal tissues. *J. Biol. Chem.* **1951**, *194*, 715−719.
(62) Bodwell, C. E., Pearson, A. M., and Fennell, R. A. Post-mortem changes in muscle III. Histochemical observation in beef and pork. *J. Food Sci.* **1965**, *30*, 944−954.

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