51st International Congress of Meat Science and Technology
August 7-12, 2005 – Baltimore, Maryland USA

MITOCHONDRIA DEPENDENT REDUCTION OF METMYOGLOBIN IN VITRO

JIALI TANG[1], CAMERON FAUSTMAN[1], and MELVIN C. HUNT[2]

[1]Department of Animal Science, University of Connecticut, 3636 Horsebarn Hill Road Ext., Storrs, Connecticut 06269-4040;
[2]Department of Animal Sciences and Industry, Kansas State University, 224 Weber Hall, Manhattan, KS 66506

Key Words: Metmyoglobin; mitochondria; reduction; NADH; NADPH

Introduction

Oxidation of ferrous myoglobin (Mb) to ferric metmyoglobin (MetMb) is responsible for fresh meat discoloration. Reduction of MetMb can delay this process and prolong desired color.

MetMb has been reported to be reduced enzymatically (DT-diaphorase, MetMb reductase) and non-enzymatically by NADH or NADPH (*1-5*). The MetMb reductase includes NADH-cytochrome b_5 oxidoreductase and mitochondrial outer membrane cytochorome b (OM cytochrome b); they are located in the mitochondrial outer membrane (*6*). Mitochondria appear to have the potential to play an important role in MetMb reduction; however, no direct reduction of MetMb by mitochondria has been demonstrated.

NADH is required for non-enzymatic and enzymatic reduction of MetMb and can be produced in cytosolic and mitochondrial fractions. Mitochondrial NADH is generated from the Krebs cycle, or by reversal of electron transport (*7-13*). Electrons from succinate or cytochrome c can be transferred to NAD^+ in the reverse direction of the electron transfer chain (ETC), and the energy required for this process can be supplied by endogenous or added ATP (*10;11*). If mitochondria are subjected to anaerobic conditions, or the ETC is inhibited at complexes III or IV, equivalents from succinate are first transferred to ubiquinone, and from there, instead of moving down the cytochrome region of the chain as in forward electron transfer, they are transferred to the NADH dehydrogenase region. Subsequently, the reduced dehydrogenase flavoprotein can reduce NAD^+ which is then available for NADH-linked reductions. This mechanism has been reported in the reduction of acetoacetate and glutamate synthesis (*14-16*). Similarly, Giddings (*17*) proposed the concept of MetMb reduction by NADH derived from reversal of electron transport in the early 1970s, but its occurrence was not demonstrated.

Collectively, mitochondria have the potential to mediate MetMb reduction by (1) providing appropriate enzyme systems, and (2) generating NADH.

Objectives

The objective of this study was to characterize potential bases for mitochondria-dependent reduction of MetMb.

Methodology

Materials and Chemicals

Beef hearts were obtained locally within 1 h of exsanguination, placed on ice and transported to the laboratory. 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% (*18*).

Bovine Mitochondria Isolation

Mitochondria were isolated from bovine cardiac muscle (2 h postmortem) according to Smith (*19*) with minor modification. Briefly, one hundred g of ground cardiac muscle was washed with 250 mM sucrose twice, and suspended in 200 ml 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 one liter with mitochondrial isolation buffer and subjected to two homogenization processes. The first of these was accomplished in a KONTES DUALL grinder (Vineland, NJ, USA) with three passes, and was followed by one pass with a WHEATON Potter-Elvehjem grinder (Millville, NJ, USA); pestles of these grinders were driven by a heavy-duty drill at 1400 rpm. The homogenate was centrifuged (1,200×g) for 20 min with a Sorvall RC-5B centrifuge (Newtown, CT, USA), and the resulting supernatant was then centrifuged (26,000×g) for 15 min. The pellet was washed twice and 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 ºC. Mitochondrial protein content was determined by a Bicinchoninic Acid Protein Assay Kit.

Reduction of MetMb

MetMb (2.5 mg/ml) was combined with different reagents in a microfuge open-top tube and incubated at 37 °C. During incubation, samples were removed and centrifuged (15,000×g) with an Eppendorf 5415D centrifuge (Westbury, NY, USA) 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 DeoMb, OxyMb, and MetMb were calculated according to Tang et al. (*18*). Mitochondrial ETC inhibitors, rotenone (0.02 mM) and myxothiaxol (0.01 mM), were used in reduction systems;

control experiments did not show any effects of these inhibitors on OxyMb oxidation or MetMb reduction (results not shown).

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 Least Square Difference (LSD) with appropriate correction for multiple comparisons (*20*).

Results & Discussion

Effect of exogenous NADH and NADPH

MetMb was reduced by NADH and NADPH non-enzymatically and the effect was concentration dependent (Figure 1; $P < 0.05$); there was no obvious reduction relative to controls when NADH or NADPH concentrations were 0.2 mM (Figure 1; $P > 0.05$). This is consistent with previous results by Brown and Snyder (*1*). In the presence of mitochondria, MetMb reduction was observed when NADH was added at 2 and 8 mM (Figure 1, $P < 0.05$), but not at 0.2 mM (Figure 1, $P > 0.05$). The addition of rotenone, an ETC complex I inhibitor, facilitated MetMb reduction in the presence of 0.2 mM NADH and mitochondria, but decreased the extent of reduction in the presence of 2 and 8 mM NADH when compared to controls without rotenone (Figure 1, $P < 0.05$). The increase in MetMb reduction with rotenone addition indicated that mitochondria-dependent reduction was more effective than non-enzymatic reduction at 0.2 mM NAD(P)H. The decreased MetMb reduction with rotenone addition suggested that NADH (2 and 8 mM) was involved in ETC-linked MetMb reduction, as previously reported (*21*).

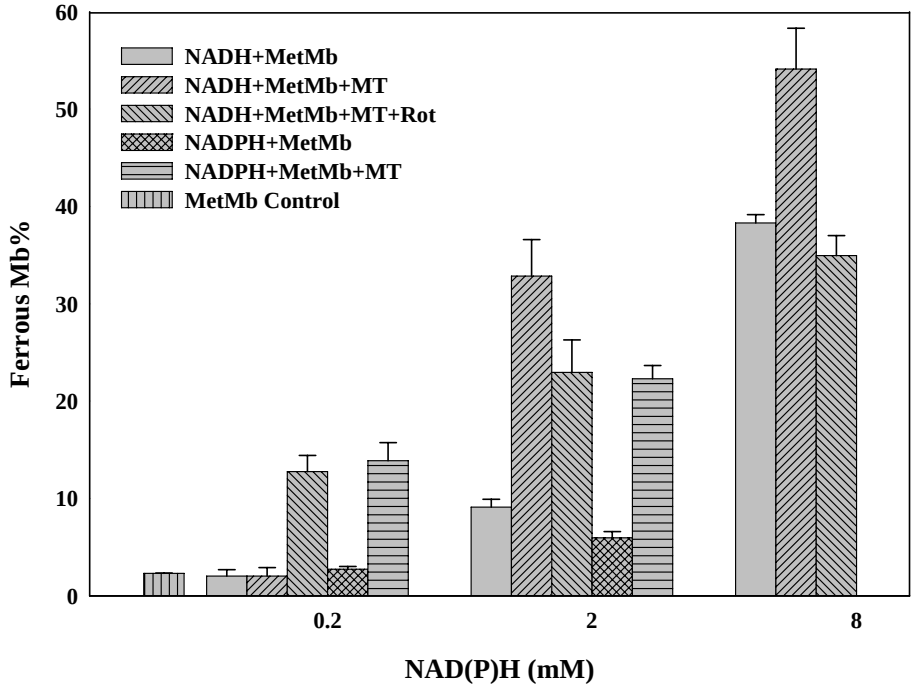

Figure 1. Reduction of metmyoglobin (MetMb, 2.5 mg/ml) by NADH or NADPH in the absence or presence of mitochondria (MT, 1.0 mg MT protein/ml) and rotenone (Rot, 0.02 mM) at 37 ºC following 1 h incubation. Controls contained MetMb in buffer only. Each system contained 120 mM KCl, 30 mM Tris-HCl, 5 mM KH_2PO_4 with a final pH 7.2.

NADPH alone or in combination with mitochondria, enhanced MetMb reduction when compared with controls and the reduction was NADPH concentration-dependent (Figure 1, $P < 0.05$). Dicumarol did not inhibit NADPH-dependent MetMb reduction, but rather increased it (Table 1; $P < 0.05$). This suggests that DT-diaphorase did not contribute significantly to mitochondria-dependent MetMb reduction by NADPH. This is not surprising given the reported lower activity of DT-diaphorase in mitochondrial fractions relative to cytosol (*22*). However, the increased MetMb reduction by dicumarol addition is not readily explained.

Table 1. The Effect of Dicumarol (0.1 mM) on NADPH-dependent Reduction of Metmyoglobin (MetMb) in the Presence of Mitochondria.*

Addition	Control	Control +Dicumarol
Ferrous Mb(%)	**12.15a**	**18.57b**

*Controls contained 120 mM KCl, 30 mM Tris-HCl, 5 mM KH_2PO_4, 0.2 mM NADPH, 2.5 mg/ml MetMb, and 1 mg mitochondrial protein/ml with a final pH 7.2; this system was incubated at 37 °C for 1 h. Means with different letters are different ($P < 0.05$).

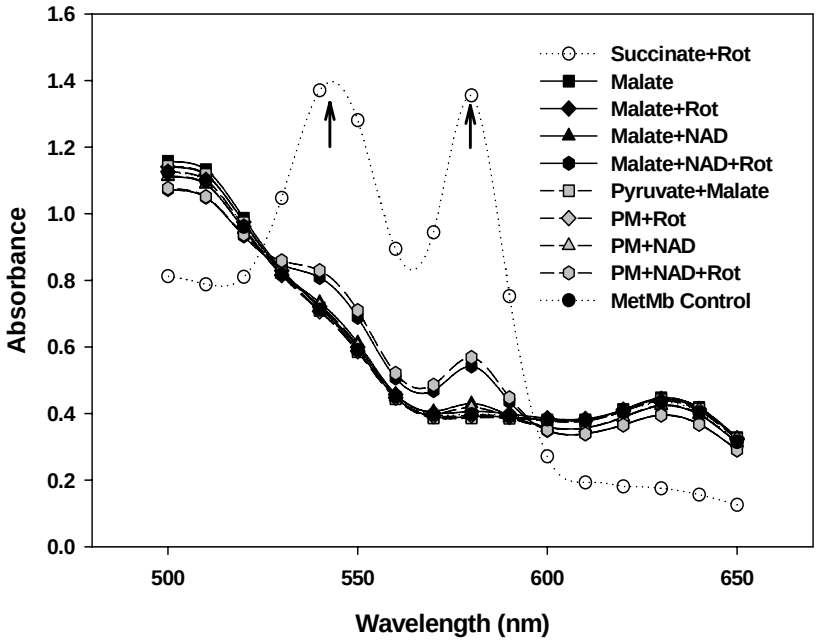

Figure 2. Metmyoglobin (MetMb) reduction by succinate (8 mM), malate (8 mM), and pyruvate (2 mM) plus malate (6 mM) (PM) in the presence of mitochondria (MT) at pH 7.2 and 37 °C for 3 h. Controls contained only MetMb in buffer. Each system contained 120 mM KCl, 30 mM Tris-HCl, 5 mM PH_2PO_4, 1.0 mg MT protein/ml, and 2.5 mg/ml MetMb. NAD^+ and rotenone (Rot) concentrations were 1.00 mM and 0.02 mM, respectively. Absorbance increases at 544 and 582 nm depicted by arrows indicates MetMb reduction.

Measured increases in absorbance values at 544 nm and 582 nm demonstrated that malate addition led to greater MetMb reduction relative to controls; however, this happened only in combination with NAD^+ and rotenone (Figure 2, $P < 0.05$). Similar results were observed with pyruvate plus malate as substrate (Figure 2, $P < 0.05$). Pyruvate and malate are NADH-linked substrates, and NAD^+ is required for production of NADH by these substrates. The requirement of rotenone for MetMb reduction to be detected in the presence of mitochondria was similar to results with 0.2 mM NADH (Figure 1), and suggested that NADH production was relatively low with NADH-linked substrates. Succinate addition accelerated MetMb reduction relative to controls (with ferrous Mb concentration) by 71.2% following 3 h incubation in the presence of rotenone (Figure 2, $P < 0.05$).

Effect of electron transfer reversal

Table 2. MetMb Reduction by Reversal of Electron Transport in Mitochondria.*

Addition	Control	Control +NAD	Control +NAD+Rot
Ferrous Mb (%)	**3.94a**	**6.22b**	**4.18a**

*Controls contained 0.25 mM sucrose, 50 mM Tris-HCl, 6 mM $MgCl_2$, 1 mM ATP, 0.01 mM myxothiazol, 8 mM succinate, 2.5 mg/ml MetMb, and 1.0 mg mitochondrial protein/ml with a final pH 7.2. The NAD^+ and rotenone (Rot) concentrations were 1 and 0.02 mM, respectively. This system was incubated at 37 ºC for 3 h. Means with different letters in the same experiment are different ($P < 0.05$).

MetMb reduction by reversed electron transport was proposed by Giddings (*17*), and Lanier and others (*3*). In order to reverse electron transport, myxothiazol, a complex III inhibitor, was added to reactions. ATP was used as an energy supply and succinate as an electron donor (*10;11*). The addition of NAD^+ increased MetMb reduction relative to controls; this reduction was abolished by the addition of rotenone, a complex I inhibitor (Table 2, $P < 0.05$). This result demonstrated that reversal of electron transport had the potential to reduce MetMb, but it is important to note that the relative effect was not great. Succinate oxidation in the Krebs cycle does not produce NADH, rather, electrons from succinate oxidation are delivered directly to ubiquinone via succinate dehydrogenase. 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. The reversal of electron transport led to decreased MetMb reduction (Table 2) when compared to forward ETC (6.2% vs. 71.2%) (Figure 2). Thus, our results suggested that forward ETC was more potentially important in mediating MetMb reduction in bovine mitochondria isolated 2 h postmortem.

Summary

Three MetMb reduction pathways mediated by mitochondria were identified: (1) rotenone insensitive and mitochondria-dependent reduction by exogenous NADH; (2) dicumarol insensitive and mitochondria-dependent reduction by exogenous NADPH; and (3) reversal of electron transport from succinate oxidation. NADH produced by oxidation of malate or malate plus pyruvate, or reversal of electron transport from succinate oxidation, also had the potential to reduce MetMb.

References

(1)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.

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

(3)Lanier, T. C.; Carpenter, J. A.; Toledo, R. T.; Reagan, J. O. Metmyoglobin reduction in beef systems as affected by aerobic, anaerobic and carbon monoxide-containing environments. J. Food Sci. 1978, 43, 1788-1792, 1796.

(4)Stewart, M. R.; K., H. B.; Zipser, M. W.; Watts, B. M. Enzymatic reduction of metmyoglobin by ground beef. J. Food Sci. 1965, 487-491.

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

(6)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.

(7)Chance, B.; Hollunger, G. Energy-linked reduction of mitochondrial pyridine nucleotide. Nature 1960, 185, 666-672.

(8)Chance, B.; Hollunger, G. The interaction of energy and electron transfer reactions in mitochondria. IV. The pathway of electron transfer. J. Biol. Chem. 1961, 236, 1562-1568.

(9)Chance, B.; Hollunger, G. The interaction of energy and electron transfer reactions in mitochondria. I. General properties and nature of the products of succinate-linked reduction of pyridine nucleotide. J. Biol. Chem. 1961, 236, 1534-1543.

(10)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.

(11)Low, H.; Vallin, I. Reduction of added DPN from the cytochrome c level in submitochondrial particles. Biochem. Biophys. Res. Commun. 1962, 9, 307-312.

(12)Hommes, F. A. The succinate-linked nicotinamide-adenine dinucleotide reduction in submitochondrial particles: II. Studies with inhibitors. Biochim. Biophys. Acta 1963, 77, 183-190.

(13)Hommes, F. A. The succinate-linked nicotinamide-adenine dinucleotide reduction in submitochondrial particles: I. Kinetic studies of the reaction. Biochim. Biophys. Acta 1963, 77, 173-182.

(14)Ernster, L.; Azzone, G. F.; Danielson, L.; Weinbach, E. C. Succinate-linked acetoacetate reduction: II. Electron transfer pathway and energy requirement. J. Biol. Chem. 1963, 238, 1834-1840.

(15)Azzone, G. F.; Ernster, L.; Weinbach, E. C. Succinate-linked acetoacetate reduction: I. Endergonic reduction of acetoacetate by succinate in liver mitochondria. J. Biol. Chem. 1963, 238, 1825-1833.

(16)Slater, E. C.; Tager, J. M. Synthesis of glutamate from alpha-oxoglutarate and ammonia in rat-liver mitochondria: V. Energetics and mechanism. Biochim. Biophys. Acta 1963, 77, 276-300.

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

(18)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.

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

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

(21)Tang, J.; Faustman, C.; Mancini, R. A.; Seyfert, M.; Hunt, M. C. Mitochondrial reduction of metmyoglobin: dependence on the electron transfer chain. J. Agric. Food Chem. 2005, In press.

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