Conversion of metmyoglobin directly to oxymyoglobin by mitochondria from pork muscle (*M. masseter)* and liver.

Erik Slinde[1,2], Vinh Phung[2] and Bjørg Egelandsdal[2]
[1] Institute of Marine Research, N-5817 Bergen, Norway
[2]Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, N-1432 Ås, Norway

Abstract— **The colour of meat is determined by the amount and state of myoglobin. The balance between electron transfer to reduce met-myoglobin at the outer mitochondrial membrane and the reduction of oxygen to water at complex IV, cytochrome c oxidase, is not well understood at present. Many phenomena take place *post mortem* that could change the present theories of met-myoglobin reduction. A better understanding of the robustness of mitochondrial complexes and the mitochondrion's structural and in particular outer membrane intactness *post mortem* are needed. We have studied mitochondrial structural changes through sedimentation experiments and through oxygen respiration protocols. Liver and muscle mitochondria metabolizing on succinate in the presence of rotenone with horse met-myoglobin added were studied. Cytochrome c was added to mitochondria and permeabilized cells respiring on succinate after blocking complex I with rotenone at different pH values. Oxygen consumption was measured by high-resolution respirometry (Oroboros Oxygraph-2K instrument). After oxygen depletion (oxygen concentration close to zero) reduction of met-myoglobin with concomitant binding of oxygen was observed as increased absorbance at 544 and 582 nm. The reduction time seemed to reflect oxygen consumption rate of the respiratory chain and not the degree of outer membrane intactness. We suggest that many cells in muscle and liver have defect outer mitochondrial membranes *post mortem,* and the outer mitochondrial cytochrome b_5 might therefore not strictly be involved in met-myoglobin reduction. The sedimentation coefficient of mitochondria from pork liver in isotonic medium vary with pH between 10-20.000 S; the larger values at low pH indicated structural modifications**

Keywords—**colour, myoglobin, mitochondria.**

I. INTRODUCTION

The colour of meat is determined by the amount of myoglobin present and its state. It is believed that the mitochondrion is important for reducing met-myoglobin. Present theories suggest that the mitochondrial complex I (NADH-dehydrogenase) and complex III (cytochrome bc_1 complex), or complex II (the succinate dehydrogenase) and complex III must remain active *post mortem* in order to achieve reduction of metmyoglobin. This again suggests that lack of substrates *post mortem* for these complexes eventually exhausts or reduces colour stability. Succinate dehydrogenase in the Krebs cycle produces $FADH_2$ by converting succinate to fumarate, thus providing electrons to the CoenzymeQ junction. The electrons assumingly travel further to the respiratory complexes III before reduction of met-myoglobin. To reduce metmyoglobin, cytochrome c is proposed to shuttle electrons from the inner to the outer mitochondrial membrane where met-myoglobin is reduced by the outer mitochondrial membrane cytochrome b_5 (Tang et al., 2005). The balance between electron transfer to reduce metmyoglobin at the outer mitochondrial membrane and the reduction of oxygen to water at complex IV, cytochrome c oxidase, is not well understood at present.

Many phenomena take place *post mortem* that could impact on the present theories of met-myoglobin reduction. First of all better understanding of the robustness of mitochondrial complexes *post-mortem* is needed. Also better understanding of mitochondrion's structural intactness, and in particular outer membrane intactness, has to be obtained.

The aim of the present study was to evaluate the reduction of metmyoglobin in the presence of succinate after blocking complex I with rotenone. Furthermore we have studied the effect of adding cytochrome c to

respiring permeabilized fibers and isolated mitochondria from pork muscle and liver. Finally we have studied the sedimentation properties of liver mitochondria at different pH values.

II. MATERIALS AND METHODS

Porcine liver and *M. masseter* muscle were removed within 1 hr of slaughter. Their location makes them easily accessible *post mortem.*

Isolation of mitochondria

Optimal recovery of mitochondria in homogeneous sucrose media used time integrals of $5.8 \cdot 10^7$ (3000 rpm) and $3.7 \cdot 10^8$ (7500 rpm) respectively, according to (Slinde et al., 1975). Glutamate dehydrogenase was used as marker enzyme.

Permeabilization of tissue

Muscle and liver tissue fibres were separated (within 3 h post mortem) in relaxing solution containing 15 mM phosphocreatine, 10 mM Ca-EGTA (0.1 mM free calcium), 20 mM imidazole, 20 mM taurine, 6.6 mM $MgCl_2$, 50 mM K-2-(*N*-morpholino) ethanesulfonic acid, 0.5 mM dithiothreitol, and 5.8 mM ATP (at pH 7.1). Both tissues were permeabilized in the same relaxing solution containing 0.052 mg/ml saponin for 30 min. Samples were subsequently washed for 10 min at 4 °C in 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-methanesulfonate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, and 1.0 g/l BSA. Chemicals were analytical grade.

Respiration measurements

High-resolution respirometry was carried out with Oroboros Oxygraph-2K (Gnaiger 2001). The protocol is shown in Table 1.

Table 1. Addition sequence of substrates and inhibitors after rotenone addition.

Symbol	Rotene(μM)	Succinate(mM)	ADP(μM)	Cytochrome c (μM)	FCCP(μM)
OCR_s	0.25	5	-	-	-
OCR_{ADP}	0.25	5	5	-	-
OCR_C	0.25	5	5	10	-
OCR_{FCCP}		5	5	10	0.5-1

Metmyoglobin reduction procedure

Isolated mitochondria (8.2 -5.5 mg/ml protein plus 1.8 mg/ml metmyoglobin were put into a medium (2 mL) consisting of 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-methanesulfonate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, and 1.0 g/l BSA. Complex I was blocked by adding 10μL 0.25 μM rotenone and then 25μL of 5 mM succinate was added and the system incubated at 20°C until oxygen was depleted. Adequate solution to fill a lidded cuvette (volume 0.8 mL) was quickly transferred and placed in a spectrophotometer (Shimadzu UV-1800 UV Spectrophotometer with UV Probe software ver. 2.33; Shimadzu corp. Kyoto, Japan) where spectra were recorded with time. The prevailing experimental conditions in the spectrophotometer did not completely preclude the presence of oxygen, so a direct transformation from met-myoglobin via myoglobin to oxymyoglobin was observed.

III. RESULTS

Table 2 . Response to cytochrome c addition.

Tissue		OCR_C/OCR_{ADP}			
		Mitochondria		Permeabilized cells	
		Muscle	Liver	Muscle	Liver
ΔOCR_C*(%)		95.2	87.4	61.5	94.2
pH	5.0	2.17**± 0.66	2.79± 1.06	1.22± 0.07	1.13± 0.09
	7.1	1.33± 0.07	2.79± 0.04	1.17± 0.19	1.05± 0.02
	7.1***	2.12± 0.22	3.80± 0.11	1.07± 0.03	1.25± 0.03

* Percentage reduction in OCR_c from pH 7 to 5**Mean values and standard errors of mean; ***Frozen and thawed samples; the data were split into fresh and frozen samples at this pH.

Permeabilized cells from liver tended to have a higher response to cytochrome c addition (P=0.06) than permeabilized muscle cells. The largest absolute variation in OCR of permeabilized cells and mitochondria following cytochrome c addition was caused by pH changes, but this was less reflected in relative values (Table 2). This was also the case for

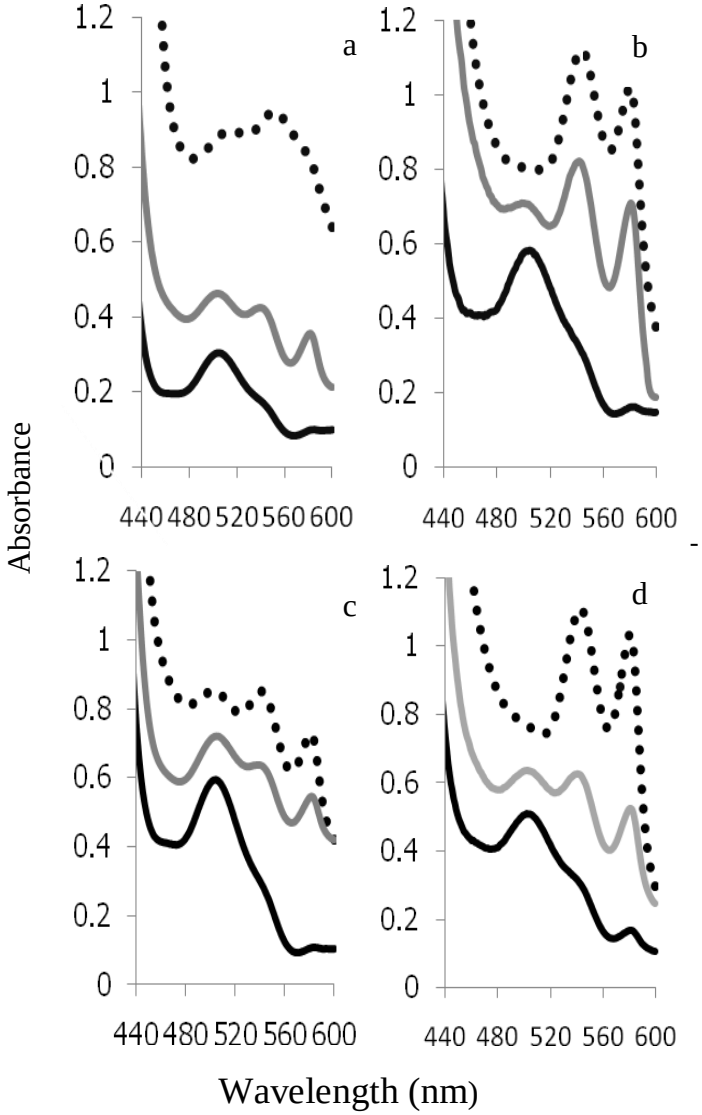

Figure 1. Isolated mitochondria respiring on succinate and met-myoglobin (1.8 mg/ml) at three different times after reaching zero pO_2 followed by interval stirring with air; a) isolated muscle mitochondria (8.2 mg/ml); b) isolated liver mitochondria (5.5 mg/ml); c) isolated muscle mitochondria frozen at -20°C and thawed; d) isolated liver mitochondria frozen at -20°C and thawed. — 0.25 h; — 3 h; ····· 20 h; The spectra were scatter corrected and shifted along y-axis.

isolated mitochondria. However, isolated mitochondria gave higher nominal responses to cytochrome c than permeabilized fibres, and in particular isolated liver mitochondria gave a larger nominal response to addition of cytochrome c (Table 2). At pH 7.1 the effect of adding cytochrome c was higher for liver mitochondria ($P<0.05$) than muscle mitochondria and higher after freezing/thawing ($P<0.05$) compared to fresh mitochondria (Table 2). Despite this observations it was difficult to detect corresponding changes in the oxygenation rate of met- to oxymyoglobin.

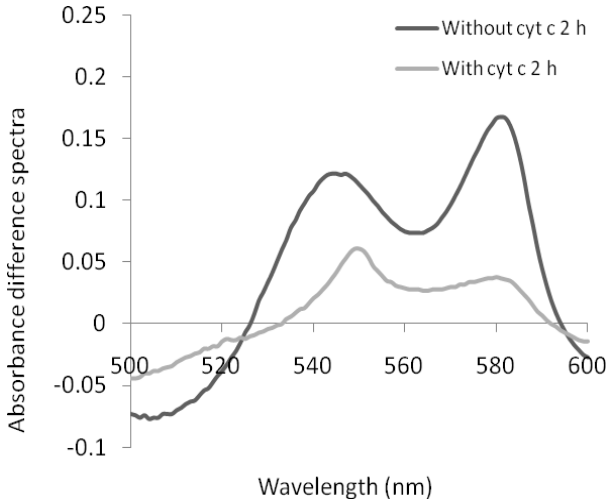

Figure 2. The difference between spectra measured 2 hrs after reaching zero pO_2 and adding cytochrome c and the spectra measured at time zero; on frozen and thawed liver mitochondria.

Figure 1 shows reduction with direct oxygenation of horse metmyoglobin for liver and muscle mitochondria metabolizing on succinate in the presence of rotenone. The reduction and oxygenation were observed after oxygen depletion (oxygen concentration close to zero) as increased absorbance at 544 and 582 nm.
The difference (substracted) spectra in Figure 2 show that addition of cytochrome c had no effect on the rate of the met-, deoxy- to oxymyoglobin conversion.

The sedimentation coefficient of mitochondria from pork liver in isotonic medium varied between 10000-20000 S depending on pH (Table 2); the larger values at low pH indicated structural modifications. At pH 5.0 the respiration of mitochondria was low (not shown) with a small absolute response to cytochrome c addition.

Table 2. Sedimentation coefficient S_{avg} (mean and standard errors of the mean) of isolated fresh liver mitochondria..				
pH	7.4	6.0	5.6	5.0
S_{avg}^{*} x 10^{-3}	10.9^{a}± 1.7**	17.9^{ab}± 1.6	20.8^{b}± 0.2	28.3^{abcd} ±3.6

* Svedberg units ;**Tukeys test

IV DISCUSSION

Tang et al. (2005) reported that equine met-myoglobin could be reduced in the presence of isolated beef heart mitochondria respiring on succinate. After 3 hrs at 37°C and at pH 7.1, direct conversion to oxymyoglobin was observed. Starting from oxymyoglobin, however, reduced myoglobin was identified at 0.5% of initial (100%) pO_2 value (Tang et al. 2005). The authors also reported that at pH 5.6 the reductions slowed down assumed due to reduced metabolic activity at the lower pH due to swelling of mitochondria as indicated by increased S_{avg} (Table 2).

Here we have used mitochondria from pork liver and *M. masseter* and equine metmyoglobin in a comparable experiment. Our pork liver mitochondria system seemed to have comparable metabolic activity to beef heart mitochondria. However, the muscle mitochondria seemed to be metabolically slower than liver mitochondria and/or possibly also less stable, *i.e.* the state oxymyoglobin was maintained for a shorter period in muscle mitochondria.

Freezing of mitochondria is accepted as a technique that disrupts the mitochondrial structure unless particular cryoprotective compounds are added. Vinh et al. (2011) reported that oxygen consumption rate became reduced with freezing at -20°C for fresh liver mitochondria frozen as pellets, while muscle mitochondria had increased oxygen consumption after freezing at -20°C and thawing. Our results gave more rapid conversion to oxymyoglobin for fresh liver mitochondria than frozen and thawed mitochondria, but the differences between fresh and frozen (as pellets) muscle mitochondria were small. Thus there were some agreement between oxygen consumption rate and metmyoglobin reduction rate.

Since it is assumed that metmyoglobin is reduced to oxymyoglobin at outer mitochondrial surface, it was interesting to consider if damages of outer mitochondrial membrane affect oxygen consumption rate. The rate of oxygen consumption was not stimulated by outer membrane damage nor was metmyoglobin reduction stimulated by cytochrome c addition.We suggest that many cells in muscle and liver have defect outer mitochondrial membranes *post mortem*, and the outer mitochondrial cytochrome b_5 may not strictly be involved in met-myoglobin reduction.

These observations suggested that shuttling of electrons to outer mitochondrial cytochrome b_5 may not necessary be involved in met-myoglobin reduction since the outer membrane can possibly be bypassed by met-myoglobin if the membrane allows cytochrome c to enter (MW 12kDa). At least that hypothesis needs further investigation.

CONCLUSIONS

Provided substrate is available, isolated muscle and liver mitochondria will eliminate oxygen in vitro and thereafter reduce met-myoglobin that rapidly binds oxygen if available. This happens even if the outer membrane is defect through isolation or freezing.

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