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ARE THERE DISTINCT SUBCELLULAR POPULATIONS OF MITOCHONDRIA IN RAINBOW TROUT RED MUSCLE?

BRENDAN J. BATTERSBY* AND CHRISTOPHER D. MOYES†

Department of Biology, Queen's University, Kingston, Ontario, Canada K7L 3N6

*Present address: Montreal Neurological Institute, 3801 University Avenue, Montreal, PQ, Canada H3A 2B4

†Author for correspondence (e-mail: moyesc@biology.queensu.ca)

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Summary

Ultrastructural analysis typically shows vertebrate striated muscles to possess mitochondria residing primarily in two locations. One population is interlaced throughout the myofibrils and another occurs directly beneath the cell membrane. The two populations of mitochondria can be separated and studied *in vitro*. Subsarcolemmal mitochondria (SSmt) are released by mechanical shearing of the tissue, whereas protease treatment is required to release the intermyofibrillar population (IMFmt). These methods were applied to rainbow trout (*Oncorhynchus mykiss*) red muscle to investigate the possible existence of distinct populations in this tissue. The two populations were very similar in mitochondrial DNA content (mtDNA mg^{-1} mitochondrial protein) and enzymatically (activities of carnitine palmitoyl transferase, β-hydroxyacyl CoA dehydrogenase, complex I, citrate synthase, cytochrome *c* oxidase expressed per milligram of mitochondrial protein). Respiration rates were the same for pyruvate and succinate, but IMFmt oxidized palmitoyl carnitine 26 % faster than SSmt ($P<0.05$). Apart from these minor differences in fatty acyl carnitine oxidation rates, no differences in biochemical or genetic properties were detected between populations. The lack of distinct subcellular populations in fish, in contrast to the situation in mammalian striated muscle, probably relates to the high mitochondrial volume density in fish red muscle.

Key words: mitochondria, subcellular population, rainbow trout, *Oncorhynchus mykiss*.

Introduction

The energetic demands of aerobic tissues such as striated muscle are met primarily using ATP generated by mitochondrial oxidation of metabolic fuels. Ultrastructural analyses demonstrate that muscle mitochondria exist not as single organelles but, instead, as a reticulum (Bakeeva *et al.* 1978). Furthermore, the reticulum is dynamic and capable of fission and fusion during differentiation, development and energetic changes (see Stevens, 1981; Bereiter-Hahn, 1990). It has been argued that the continuous reticular structure offers advantages for electrical conductance (Amchenkova *et al.* 1988) and in the diffusivity of O_2 (see Bereiter-Hahn, 1990).

In contrast to this view of a mitochondrial syncytium, a number of studies have shown at least two distinct populations of mitochondria within striated muscle: one directly beneath the sarcolemma (SSmt), and a second interlaced amongst the myofibrils (intermyofibrillar, IMFmt). Theoretical arguments have been made for the physiological benefits of particular locations with respect to ATPases and gradients of oxygen and blood-borne fuels (see Cogswell *et al.* 1993). Morphological studies suggest that the two populations have different susceptibilities or responsiveness to physiological challenges *in vivo* (e.g. Muller, 1976; Kreiger *et al.* 1980; Howald *et al.* 1985; Duan and Karmazyn, 1989). Separation of the populations in both cardiac (Palmer *et al.* 1977) and skeletal (Krieger *et al.* 1980) muscles relies upon shearing to release SSmt and subsequent protease treatment to release IMFmt. Despite early concerns (Matlib *et al.* 1981; Palmer *et al.* 1981), the distinct populations do not appear to arise as isolation artefacts (Palmer *et al.* 1985). Intermyofibrillar mitochondria typically demonstrate as much as threefold higher rates of respiration with a variety of substrates in both cardiac (Palmer *et al.* 1977) and skeletal (Cogswell *et al.* 1993; Krieger *et al.* 1980) muscle. Recent studies have focused on the possible origins and maintenance of distinct properties of mitochondrial populations from the perspective of protein import and *in organello* protein synthesis. Studies on muscle (Cogswell *et al.* 1993; Takahashi and Hood, 1996) and other tissues (Lopez-Mediavilla *et al.* 1995; Cossarizza *et al.* 1996) suggest that subcellular population differences may reflect mitochondria at different stages of biogenesis.

Although there is considerable evidence for the existence of functionally distinct subcellular populations of mitochondria in striated muscle of mammals, studies in other species are limited. Fish have been used in studies of muscle function because red and white muscle are well separated anatomically and differ in oxidative capacity by as much as 10-fold (see

Johnston and Moon, 1981). In comparison with mammalian oxidative muscle, fish red muscle has a much higher mitochondrial volume density (Johnston and Moon, 1981; Moyes *et al.* 1992) and cristae packing density (Egginton and Sidell, 1989; Moyes *et al.* 1992). There is also a striking diversity of mitochondrial fuel preferences across fish species (see Moyes *et al.* 1990). In the present study, we investigated the possible existence of subcellular populations of mitochondria from salmonid red muscle using enzymatic, respiratory and genetic indices.

Materials and methods

Animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] were obtained from Pure Springs Trout Farms in Shannonville, Ontario, Canada. All fish, weighing approximately 1 kg, were kept in a flow-through tank at 4 °C for 2 months under a 12 h:12 h light:dark photoperiod regime.

Mitochondrial populations

Subcellular populations of mitochondria were isolated using a modification of the technique of Cogswell *et al.* (1993). It is difficult to demonstrate directly that the method specifically yields pure fractions of SSmt and IMFmt as there is no 'marker' of intracellular location. In most studies with fish muscle, mitochondria are generated by mechanical shearing and would be analagous to the SSmt fraction in the present study. Red muscle was minced on iced glass then transferred to 10 vols of buffer A (140 mmol l^{-1} KCl, 5 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} Hepes, 10 mmol l^{-1} EDTA, pH 7.0) with 0.5 % bovine serum albumin (BSA). Samples were homogenized with two passes of a loose-fitting Teflon pestle followed by three passes with a tight-fitting pestle. Homogenates were then centrifuged at 800 ***g*** for 10 min at 4 °C to separate the supernatant (containing SSmt) from the myofibrils (containing IMFmt).

Subsarcolemmal mitochondria

The resulting supernatant was filtered through eight layers of cheesecloth and centrifuged for 10 min at 9000 ***g***. SSmt pellets were washed twice by resuspension in buffer A and recentrifugation.

Intermyofibrillar mitochondria

The myofibril pellet was gently resuspended in buffer A (+BSA), then recentrifuged to wash away mitochondria loosely associated with the myofibrils. The supernatant was discarded, and the pellet was resuspended in buffer A containing 4 mg of Neutralase (ICN) for each gram of original tissue. After a 30 min digestion on ice, an equal volume of buffer A (+BSA) was added, and the suspension was then centrifuged for 5 min at 5000 ***g***. The resulting pellet was resuspended in buffer A and centrifuged (10 min at 800 ***g***) to remove partially digested myofibrils. The supernatant was filtered through eight layers of cheesecloth and centrifuged at 9000 ***g*** for 10 min. The pellet was then washed twice in buffer A. Both IMFmt and SSmt pellets were resuspended in buffer A to approximately 5 mg protein ml^{-1}.

Mitochondrial rates of oxygen consumption

Respiration studies were performed as described previously (Moyes *et al.* 1992). Approximately 50 µl of IMFmt and SSmt suspensions were incubated in 2 ml of respiration medium (140 mmol l^{-1} KCl, 20 mmol l^{-1} Hepes, 5 mmol l^{-1} Na_2HPO_4 and 0.5 % BSA, pH 7.3). Rates of oxygen consumption were monitored polarographically at 5 °C using a Clark-type electrode interfaced with Vernier Instruments data logger software. The state 4 rate was determined for each mitochondrial preparation by the addition of saturating concentrations of one of the following substrates: pyruvate (5 mmol l^{-1}), palmitoyl carnitine (60 µmol l^{-1}) or succinate (10 mmol l^{-1}), together with 0.1 mmol l^{-1} malate for a period of approximately 5 min. State 3 respiration was stimulated by the addition of 0.2 mmol l^{-1} ADP. Respiratory control ratios (RCRs), the ratio of the state 3 rate to the state 4 rate, were determined for each mitochondrial preparation as an index of mitochondrial quality.

Enzyme activities

Maximal enzyme activities were determined under optimal conditions using a Molecular Devices Spectra Max 250 spectrophotometer at 25 °C.

Carnitine palmitoyl CoA transferase (CPT) (EC: 1.9.3.1): activity was measured in a reaction mixture containing 20 mmol l^{-1} Tris, pH 8.0, 0.2 mmol l^{-1} dithio-bis(2-nitrobenzoic acid) (DTNB), 0.1 mmol l^{-1} palmitoyl CoA and 5 mmol l^{-1} carnitine. The rate of change in absorbance was monitored at 412 nm. Controls, lacking carnitine, were run simultaneously in parallel wells, and these rates were subtracted from the rate in the presence of carnitine. The detergent treatment of the homogenate probably inactivates the outer CPT isoform (CPT I). Consequently, this assay would be expected to measure only the inner CPT II isoform, which is measured in the physiologically relevant direction (palmitoyl carnitine production). This is also the direction and isoform utilized in the respiratory rate measurements using palmitoyl carnitine as substrate.

Citrate synthase (CS) (EC: 4.1.3.7): activity was measured in a reaction mixture containing 20 mmol l^{-1} Tris, pH 8.0, 0.2 mmol l^{-1} DTNB, 0.3 mmol l^{-1} acetyl CoA, 0.05 % Triton X-100 and 0.5 mmol l^{-1} oxaloacetate. The rate of change in absorbance was monitored at 412 nm. Controls, lacking oxaloacetate, were run simultaneously in parallel wells, and these rates were subtracted from the rate in the presence of oxaloacetate.

Complex 1 (NADH dehydrogenase) (EC: 1.6.99.3): activity was measured in a reaction mixture containing 25 mmol l^{-1} potassium phosphate buffer, pH 7.4, 0.05 mmol l^{-1} dichlorophenol-indophenol, 0.1 mmol l^{-1} NADH and 0.01 % Triton X-100.

Cytochrome *c* oxidase (COX) (EC: 2.3.1.21): activity was measured in a reaction mixture containing 20 mmol l^{-1} Tris, pH

8.0, 0.5 % Tween 20 and 0.05 mmol l^{-1} reduced cytochrome *c*. Sample homogenates were preincubated for 6 min at 15 °C in the presence of the complete assay mixture prior to the addition of cytochrome *c*. Shorter incubation periods resulted in lower activities. The rate of change in absorbance was monitored at 550 nm.

β-Hydroxyacyl CoA dehydrogenase (HOAD) (EC: 1.1.1.35): activity was measured in a reaction mixture containing 50 mmol l^{-1} imidazole, pH 7.4, 0.15 mmol l^{-1} NADH, 0.1 % Triton X-100 and 0.1 mmol l^{-1} acetoacetyl CoA. Absorbance changes were monitored at 340 nm. Controls (lacking acetoacetyl CoA) did not result in changes in absorbance.

Quantitative competitive (QC) polymerase chain reaction

The mitochondrial DNA content of isolated mitochondria was determined using a quantitative competitive polymerase chain reaction (PCR) (Piatak *et al.* 1993; Zimmermann and Mannhalter, 1996). Reactions are run with samples containing both the DNA of interest (mtDNA) and an internal control. Both the control and the unknown DNA use the same primers, but amplification of the control yields a unique product. In our case, a deletion mutant results in a shorter product. The ratio of the amount of target DNA (mtDNA) to the amount of the competitor can be used to give an estimate of the amount of mtDNA. The internal standard eliminates potential tube-to-tube variation that can occur with PCR reactions.

A competitor template was made by removing an internal 260 bp *Sac*II fragment from a 603 bp fragment of trout 16S cDNA cloned into the plasmid pCRII (construct labelled as pCM45). After gel purification, the digested plasmid was ligated overnight using T4 DNA ligase (Promega) creating the Δ16S deletion mutant (pCM46). Ligated plasmids were transfected into the *Escherichia coli* strain XL-1 Blue (Stratagene) and sequenced to confirm the 260 bp deletion from the pCM45.

Mitochondrial DNA for each sample was amplified from mitochondrial suspensions in triplicate using Taq polymerase (Pharmacia) in the following reaction mixture: 10 mmol l^{-1} Tris–HCl, pH 9.0, 1.5 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} KCl, 800 μmol l^{-1} dNTP and 220 ng of primers 1 (5′-AACATCGCCTCTTGCAAATCA-3′) and 2 (5′-CCGGTCTGAACTCAGATCACGT-3′). The appropriate amount of competitor template was determined from preliminary studies and identified as that resulting in equal amounts of 16S and Δ16S product (taking into account the difference in molecular mass).

Mitochondrial suspensions were freeze-thawed four times to rupture the membrane and release mtDNA (Wang *et al.* 1994). Amplifications were performed using an initial denaturation (95 °C, 4 min) and 20 cycles of denaturation at (95 °C, 45 s), annealing (60 °C, 90 s) and extension (72 °C, 60 s). PCR products were separated electrophoretically on a 1.5 % agarose gel and stained with 0.5 μg ml^{-1} ethidium bromide to distinguish between the amplified 16S (603 bp) and the Δ16S (343 bp) fragments. A Polaroid 665 negative of the ethidium-bromide-stained gel was used to quantify PCR products using a Molecular Dynamics computing densitometer with ImageQuant (version 3.3) software.

Results and discussion

In the present study, little evidence for substantial differences in mitochondrial populations was found. No differences between populations were detected in state 3 respiration rates for either pyruvate or succinate (Table 1). Maximal enzyme activities were measured on the same mitochondrial preparations used in the respiration studies to determine whether differences existed in the profile of mitochondrial enzymes between the two populations. None of the enzymes demonstrated any significant difference in activity (nmol mg^{-1} mitochondrial protein min^{-1}) or in enzyme ratios between IMFmt and SSmt (Table 2). Similarly, mtDNA content (ng mg^{-1} mitochondrial protein) did not differ between populations (Fig. 1).

Although little evidence for dramatic differences in mitochondria were found in rainbow trout, this does not preclude the possibility that otherwise identical populations may be differentially regulated *in vivo*. As has been argued in mammalian studies (see Cogswell *et al.* 1983; Takahashi and Hood, 1996), different subcellular locations may allow differential access to mitochondrial substrates. A subsarcolemmal location could permit preferential access to blood-borne oxygen or carbon fuels. A myofibrillar location mighty reduce diffusion distances for adenylates (ATP, ADP) and phosphate, particularly during periods of muscular activity.

Although there were no significant differences between populations in the respiration rates for either pyruvate or succinate, IMFmt oxidized palmitoyl carnitine significantly (26 %; $P<0.05$) faster than did SSmt. Paradoxically, no differences were detected between populations in the activity of CPT or HOAD (Table 2). Enzymatic differences between mitochondrial populations do not generally correlate with respiratory differences in either magnitude or direction. For example, Cogswell *et al.* (1993) showed that IMFmt have 2.8-

Table 1. *Substrate oxidation by isolated mitochondrial populations from rainbow trout skeletal muscle*

	Intermyofibrillar mitochondria	Subsarcolemmal mitochondria
Yield (μg protein g^{-1} tissue)	471.6±79.9	471.5±63.7
Respiratory control ratio	11.89±1.37	11.19±1.65
Oxidation rates (nmol O_2 min^{-1} mg^{-1} protein)		
Pyruvate	30.19±1.87	28.01±2.96
Palmitoyl carnitine	37.36±1.86	29.76±2.83*
Succinate	36.26±2.15	31.41±2.88

Values are means ± S.E.M., $N=8$.

*A value significantly different (paired Students *t*-test) for the two populations ($P<0.05$).

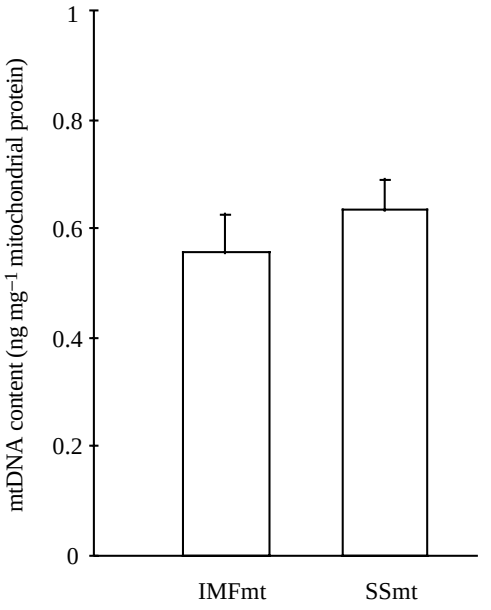

Fig. 1. Comparison of mitochondrial DNA (mtDNA) contents in isolated subsarcolemmal (SSmt) and intermyofibrillar (IMFmt) mitochondria from rainbow trout red muscle. The content of mtDNA was estimated using quantitative competitive polymerase chain reaction (QC-PCR). Values are means ± S.E.M., *N*=9.

fold higher respiratory rates, but that COX activity was only 20 % greater and succinate dehydrogenase activity 40 % lower. The observed differences in the capacity of IMFmt and SSmt to oxidize fatty acyl carnitine is reminiscent of comparisons between mitochondria prepared from different fibre types (see Moyes *et al.* 1990, 1992).

To understand why distinct populations might not be observed in trout red muscle, it is important to consider how mammalian muscle generates biochemically distinct subcellular mitochondrial populations. A number of studies have examined the maintenance of genetic heterogeneities in single cells. Work with cybrids (enucleated cells fused with nucleated cells) has shown that cells possessing two distinct mitochondrial populations can demonstrate environmentally induced shifts in subcellular populations (Tonsgard *et al.* 1990). Focal changes in mtDNA occur in myopathic conditions in which mtDNA possessing large deletions accumulates, possibly through a replicative advantage (Grossman and Shoubridge, 1996). In the absence of intracellular genetic heterogeneities, it is less clear how distinct populations arise and are maintained. When transcribed on cytosolic free ribosomes, mitochondrial pre-proteins are translated and become available for uptake by mitochondria. There is little evidence that any process from nuclear transcription to cytosolic translation could benefit one mitochondrial population over another. Where subcellular differences have been demonstrated, there is abundant evidence that the mitochondria themselves have some control over the post-translational uptake and processing of cytoplasmically located mitochondrial pre-proteins (Takahashi and Hood, 1996).

Table 2. *Mitochondrial enzyme activities of isolated mitochondrial populations in rainbow trout red muscle*

	Intermyofibrillar mitochondria	Subsarcolemmal mitochondria
Enzyme activities		
Citrate synthase (CS)	1793±120	1975±116
Cytochrome *c* oxidase (COX)	1703±110	1876±157
Complex I (COM-I)	93.3±6.5	94.4±6.2
Carnitine palmitoyl CoA transferase (CPT)	106±8.6	108.3±7.0
β-hydroxyacyl CoA dehydrogenase (HOAD)	1821±144	1818±155
Enzyme ratios		
COX/CS	0.977±0.092	0.965±0.093
COM-1/CS	0.053±0.004	0.049±0.004
CPT/CS	0.059±0.003	0.055±0.003
HOAD/CS	1.015±0.037	0.922±0.059

Values are given as mean ± S.E.M., *N*=9.
Activites are expressed nmol substrate min^{-1} mg^{-1} mitochondrial $protein^{-1}$.

Protein transport, folding and assembly are all energy-dependent processes, and there is considerable evidence that subcellular differences in mitochondrial bioenergetics occur (e.g. Smiley *et al.* 1991). At the level of mitochondrial biogenesis, differences in molecular chaperone levels, bioenergetics and import of both proteins and regulatory factors may all contribute to mitochondrial heterogeneities within cells (Takahashi and Hood, 1996).

Although these factors could contribute to the generation and maintenance of heterogeneous subcellular populations of mitochondria, mechanisms also exist that would tend to reduce heterogeneities. Muscle mitochondria typically occur as a reticulum that undergoes continuous fusion and fission, processes that would be expected to reduce mitochondrial heterogeneities. Thus, the kinetics of mitochondrial reticulum formation would be expected to influence the extent of subcellular differences in mitochondrial properties. In this regard, fish muscle shows important morphological differences from mammalian muscle. At the ultrastructural level, fish skeletal muscle has a high mitochondrial volume density and high cristae packing density. The red muscle of fish, in general, possesses mitochondrial volume densities of 25–45 % (e.g. Johnston and Moon, 1981; Johnston *et al.* 1997), well in excess of those of human skeletal fibres (e.g. <6 %; Howald *et al.* 1985) and even approaching those of cardiac muscle. It is likely that the absence of subcellular populations of mitochondria is related to the high mitochondrial volume density in fish muscle. Equine muscle has a high mitochondrial content by mammalian standards, with a mitochondrial reticulum that extends from the subsarcolemmal region into the myofibrils (Kayar *et al.* 1988), suggesting that the maintenance of distinct populations might be a function of mitochondrial content (Cogswell *et al.* 1993).

In fish, both endurance exercise (Farrell *et al.* 1991) and cold

acclimation (Egginton and Sidell, 1989) increase muscle mitochondrial enzyme levels by 30–75 %, but the relative effects on SSmt *versus* IMFmt are not known. A number of pathophysiological factors have been shown preferentially to affect SSmt (Muller, 1976; Krieger *et al.* 1980; Howald *et al.* 1985; Duan and Karmazyn, 1989). Exercise training has a greater relative effect on SSmt content, but these mitochondria typically represent approximately 25 % of the total mitochondrial pool (e.g. Howald *et al.* 1985). It has been argued that the subcellular populations may reflect mitochondria at different stages of biogenesis (Takahashi and Hood, 1996). The high mitochondrial content in fish may also hide the existence of a much smaller proportion of mitochondria at earlier stages of biogenesis.

In summary, the high mitochondrial content of fish muscle may preclude the maintenance of subcellularly distinct mitochondrial populations. The evolutionary origins, mechanistic basis and regulatory consequences of the high mitochondrial content and cristae surface area in fish are largely unknown.

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