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Isolation of mitochondria for biogenetical studies: An update

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ABSTRACT

The use of good quality preparations of isolated mitochondria is necessary when studying the mitochondrial biogenetical activities. This article explains a fast and simple method for the purification of mammalian mitochondria from different tissues and cultured cells, that is suitable for the analysis of many aspects of the organelle's biogenesis. The mitochondria isolated following the protocol described here, are highly active and capable of DNA, RNA and protein synthesis. Mitochondrial tRNA aminoacylation, mtDNA–protein interactions and specific import of added proteins into the organelles, can also be studied using this kind of preparations.

1. Introduction

Mitochondria are key organelles as their main function is to synthesize most of the cell's ATP via the oxidative phosphorylation (OXPHOS) system. In addition, they are involved in many other metabolic pathways including nucleotide precursor biosynthesis, cofactor biosynthesis, metal ion homeostasis and are also central in the apoptotic process.

Animal mitochondria contain their own genome in which 13 polypeptides, all of them structural subunits of the OXPHOS Complexes I, III, IV and V, are encoded. The information for the rRNAs and tRNAs necessary for the translation of these polypeptides within the organelle is also contained in the mitochondrial DNA (mtDNA). The rest of the complexes structural subunits and the factors involved in mtDNA expression, as well as many other proteins with diverse functions, are encoded in the nucleus and must be imported inside mitochondria. Mitochondrial dysfunction, of mitochondrial or nuclear genetic origin, is the cause of human disorders with a wide range of clinical presentations (Zeviani and Carelli, 2007; Zeviani and Di Donato, 2004). Moreover, malfunction of mitochondria has been also associated to neurodegenerative diseases (Petrozzi et al., 2007), diabetes (Mulder and Ling, 2008) and cancer (Kroemer, 2006). However, the mechanisms involved in all these diseases are to a great extent still unknown. The availability of quality mitochondrial preparations is very useful in the study of the processes underlying these pathologies as well as in the basic knowledge of mitochondrial functionality.

The use of isolated mitochondria has proved to be a very powerful tool in the study of the OXPHOS system biogenesis under conditions resembling the *in vivo* enzyme/substrate proportions, ionic composition, and integrated activity of the metabolic processes (Enriquez et al., 1999b, 1996b). In addition, they allow a better control and manipulability of the experimental conditions than when using intact cells or tissues. Isolated mitochondria preserve the ability to synthesize DNA, RNA and proteins. This capacity has been shown to be predetermined by the metabolic state of the cell before the mitochondria were isolated like, for example, by the thyroid state of the animal (Enriquez et al., 1999a; Fernandez-Vizarra et al., 2008).

Many procedures for the isolation of mammalian mitochondria from different sources are available in the literature and some have been recently reviewed (Frezza et al., 2007; Pallotti and Lenaz, 2007). These methods are similar to the one presented herein, which we have been using successfully for some years now. The protocol described here, which has not been modified significantly from the one published previously (Fernandez-Vizarra et al., 2002), is simple and rapid and yields reasonably pure, intact and highly

Abbreviations: MtDNA, Mitochondrial DNA; mtRNA, Mitochondrial RNA; OXPHOS, Oxidative phosphorylation; COX, Cytochrome c oxidase; CS, Citrate synthase; CCCP, Carbonyl cyanide m-chlorophenylhydrazone.

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functional mitochondria from different mammalian tissues and cultured cells, proved to be suitable for the analysis of the different processes involved in the biogenesis of the OXPHOS system like mtDNA replication (Enriquez et al., 1994; Garrido et al., 2008; Gensler et al., 2001), transcription (Enriquez et al., 1999a, 1996a; Fernandez-Vizarra et al., 2008; Garrido et al., 2008; Garstka et al., 2003; Viscomi et al., 2009), tRNA aminoacylation (Enriquez and Attardi, 1996b) and translation (Fernandez-Silva et al., 2007; Fernandez-Vizarra et al., 2008), as well as protein import (for example: (Fernandez-Silva et al., 1997; Fernandez-Vizarra et al., 2007a; Ghezzi et al., 2008; Petruzzella et al., 1998)). Mitochondrial fractions prepared in this way have been also used for enzymatic activity measurements (Fernandez-Vizarra et al., 2006; Moreno-Loshuertos et al., 2006) and to study the interaction of proteins with mtDNA (Enriquez et al., 1999a; Micol et al., 1997). The general procedure can be used with little modifications depending on the source of mitochondria and it is based on differential centrifugation, not making use of any kind of density gradient. When the crude mitochondrial fraction is obtained, it is then washed several times by centrifugation in order to eliminate a considerable fraction of contaminants (Table 1) (Fernandez-Vizarra et al., 2006).

Since the protocol was published in a methods journal some years ago (Fernandez-Vizarra et al., 2002), it has been used by several research groups to obtain high quality mitochondrial preparations to perform various types of functionality studies. In our experience, mitochondrial preparations from diseased cells and tissues show the same yield and quality as the ones from normal samples (Massa et al., 2008; Perales-Clemente et al., 2008; Viscomi et al., 2009). In this paper we will describe the isolation method updating the kind of biogenetical analysis that can be performed. Using the mitochondrial fractions isolated using our protocol (see below and Figs. 1 and 2) it is possible to perform the well known mitochondrial DNA, RNA and protein synthesis, tRNA aminoacylation and protein import analyses. In addition, the combination of the *in organello* protein synthesis and the protein import assays with Blue-Native Gel Electrophoresis allows one to follow the incorporation of newly synthesized mtDNA-encoded subunits or imported nuclear subunits into the OXPHOS complexes (Figs. 4D and 5).

2. Isolation of mitochondria from mouse and rat tissues

2.1. Mitochondria from liver and kidney

Sterile solutions and lab ware are used and once the organs are removed from the animals, all steps are performed at 4 °C or on ice. The animals used are either adult Wistar rats (weighing 200–300 g) or C57BL/6 J or NZB mice (weighing 25–30 g) which are sacrificed by decapitation or cervical dislocation, respectively. For a schematic explanation of the procedure see Fig. 1A. The organs of interest are extirpated (the gallbladder is removed from the mouse liver), rinsed using cold homogenization medium and immediately placed in the homogenization medium A (0.32 M sucrose, 1 mM EDTA, and 10 mM Tris–HCl, pH 7.4) previously cooled inside a beaker on ice. Once the organs are weighed, they are cut into small pieces using a pair of scissors and are washed to remove blood and connective tissue. This is achieved using a metal sieve and adding fresh homogenization medium until the tissue is well cleaned (repeating this step 3–4 times). Subsequently, fresh homogenization medium A is added to a glass Elvehjem potter together with the cut and clean tissue, in a proportion of 4 ml/g of liver or 5 ml/g of kidney. The homogenization is performed using a motor-driven Teflon pestle with 4 up and down strokes at 600 rpm. The homogenate is then transferred to a 50 ml centrifuge tube that is spun at 1000*g* for 5 min at 4 °C to sediment unbroken tissue, cells and nuclei. The supernatant (S1) is transferred to eight 1.5 ml-Eppendorf tubes, previously cooled down on ice, centrifuged in a microfuge at 15,000*g* (~13,000 rpm in an Eppendorf microfuge) during 2 min at 4 °C. The supernatant is removed, carefully eliminating all the fat and the light colored fluffy layer on the top of the darker pellet containing the mitochondria. At this point, homogenization medium is added into half of the tubes so that the contents of two Eppendorf tubes are combined into a single one and resuspended together in 1.5 ml of medium. Mitochondria are resuspended very gently using glass Pasteur pipettes with a bulb. These are spun down again under the same conditions and the procedure is repeated until one single pellet is obtained. The final pellets are equilibrated in 1 ml of the appropriate buffer for the experiment to be performed, pelleted again, and resuspended in 1 ml of the final buffer. The resuspended mitochondria are maintained in a water–ice bath until used. In the case of liver and kidney mitochondria, the direct transfer of the S1 supernatant to the Eppendorf tubes yields enough mitochondria for most purposes. This series of washes are efficient for the elimination of a significant amount of contaminating particles that contain high concentrations of harmful enzymes such as nucleases, phospholipases and proteases (Fernandez-Vizarra et al., 2006), being particularly important in the isolation of liver and kidney mitochondria when prepared to analyze or purify mitochondrial RNAs.

Typically, a 250-g rat has around 9 g liver, and 2–3 g kidney (two). A 30 g mouse has an approximately 1.5–2 g liver; therefore, to have at least 5 g of starting material, 3 or 4 mice need to be sacrificed, although a 2-g liver can yield enough mitochondria for some purposes. The yield of these mitochondrial preparations with respect to the wet weight of starting tissue is estimated as approximately 6 mg mitochondrial protein per g of liver or kidney.

2.2. Mitochondria from heart

The protocol to extract heart mitochondria (Fig. 1B) begins following the same steps described above. However, the homogenization medium utilized is medium AT (0.075 M sucrose, 0.225 M

Table 1
Yield of mitochondria and some contaminant particles[a] in liver and kidney crude mitochondrial preparations (P2 pellet) and washed mitochondria: which are the preparations obtained using the washing steps in Eppendorf tubes as described in the text and in Fig. 1.

	Liver		Kidney	
	Crude fraction (P2)	Washed mitochondria	Crude fraction (P2)	Washed mitochondria
Yield of mitochondria (% of recuperation of total COX activity)	22.2 ± 4.5	23.2 ± 0.6	34.0 ± 7.5	19.1 ± 2.7
Yield of mitochondria (% of recuperation of total CS activity)	24.7 ± 2.2	27.2 ± 0.2	27.3 ± 2.1	13.7 ± 0.4
Yield of microsomes (% of recuperation of total Glucose-6-Phosphatase activity)	20.1 ± 6.1	7.9 ± 1.7	11.6 ± 1.7	3.5 ± 0.8
Yield of lysosomes (% of recuperation of total Acid Phosphatase activity)	24.5 ± 1.9	14.2 ± 2.6	14.5 ± 0.7	7.2 ± 2.9
Yield of peroxisomes (% of recuperation of total Catalase activity)	13.5 ± 4.8	4.1 ± 1.3	7.7 ± 3.0	3.1 ± 1.2

[a] Data from Fernandez-Vizarra et al. (2006).