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## Cytochrome *c* oxidase assay in minute amounts of human skeletal muscle using single wavelength spectrophotometers

Òscar Miró<sup>a</sup>, Francesc Cardellach<sup>a,\*</sup>, Antoni Barrientos<sup>a</sup>, Jordi Casademont<sup>a</sup>, Agnès Rötig<sup>b</sup>, Pierre Rustin<sup>b</sup>

<sup>a</sup> *Muscle Research Unit, Department of Internal Medicine, \*\*IDIBAPS, Hospital Clinic, University of Barcelona, Villarroel 170, 08036 Barcelona, Spain*

<sup>b</sup> *Unité de Recherche sur les Handicaps Génétiques de l'Enfant (INSERM U393), Hôpital des Enfants Malades, Paris, France*

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### Abstract

Determination of cytochrome *c* oxidase (COX; EC 1.9.3.1) activity in human mitochondria presents several technical difficulties which result in a large intra- and interlaboratory variability, especially when a single wavelength spectrophotometer (SWS) is used, as is generally done in most laboratories in the context of screening procedures for the detection of respiratory chain deficiencies. We studied the experimental conditions of COX assay in human skeletal muscle mitochondria using a SWS in order to define the optimal conditions for the assay and compared these results with those obtained using a double wavelength spectrophotometer (DWS). We demonstrate that a low intra-individual variability of COX assay can be obtained with SWS by: (1) using manual stirrers to avoid the formation of bubbles in the mixture; (2) preincubating mitochondria and laurylmaltoside before the addition of cytochrome *c*, which prevents light scattering secondary to mitochondrial swelling; and (3) using low amounts (1–2  $\mu\text{g}$ ) of mitochondrial protein to extend and linearize the reaction rate. Under these experimental conditions, the concordance between SWS and DWS was very good ( $R = 0.975$ ). © 1998 Elsevier Science B.V. All rights reserved.

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### 1. Introduction

Assay of mitochondrial enzyme activities in human skeletal muscle has proved to be a difficult task, due in part to the small amount of available material and also to contamination of the mitochondrial fractions (Cooperstein and Lazarow, 1951). As a result, amazing variations in the specific activity of respiratory chain complexes (Trounce et al., 1989, Schoffner et al., 1991, Cardellach et al., 1993, Rustin et al., 1994, Barrientos et al., 1996, Brierley et al., 1996) may sometimes lead to the diagnostic value of some of these determinations being questioned.

Determination of cytochrome *c* oxidase (COX; EC 1.9.3.1) activity presents additional difficulties due to the rapid inhibition of the enzyme by its reaction product, oxidized cytochrome *c*. Indeed, the  $K_i$  of the enzyme for oxidized cytochrome *c* does not significantly differ from the  $K_m$  of the enzyme for its substrate, reduced cytochrome *c* (Rustin et al., 1991). Determination of the enzyme activity therefore has to be calculated on the initial rate of the reaction, which is usually hard to estimate because of the rapidly decreasing rates. On the other hand, the mitochondrial outer membrane constitutes a physical barrier for cytochrome *c* which has to be destroyed in order to measure the enzyme activity fully. This has been shown to be easily achieved using a detergent; laurylmaltoside is generally favoured because of its low toxicity for COX (Kadenbach et al., 1986). However, large changes in the light scattering occurring in organelle suspensions after detergent addi-

\* Corresponding author. Tel.: +34 3 2275539; fax: +34 3 4515272; e-mail: cardell@d4.ub.es

\*\* IDIBAPS: Institut d'Investigacions Biomèdiques August Pi i Sunyer.

tion cause major interference with the determination of the initial activity rates when a single wavelength spectrophotometer (SWS) is used. In addition, changes in optical density due to the presence of foam and bubbles induced by mechanical stirring of small volume cuvettes (1 ml) with no place for a stir bar can also bring additional difficulties to the initial rate determination. Use of a double wavelength spectrophotometer (DWS) is to be preferred because it overcomes most of the above problems. However, as DWSs are quite expensive, only SWSs are generally available in screening laboratories.

In recent years, we (Rustin et al., 1991, 1994, Chretien et al., 1994) and others (Zheng et al., 1990, Birch-Machin et al., 1994, Shanske and Di Mauro, 1994, Trounce et al., 1996) have tried to improve and standardize the experimental conditions for these assays with the aim of making them a practical and workable tool in the context of the screening procedures for respiratory chain defects in human diseases. We therefore re-investigated the conditions for COX assay with the aim of devising a simple and reproducible method that would avoid difficult and complex calculations in the determination of initial rates, and would also be workable with a SWS.

## 2. Materials and methods

### 2.1. Preparation of mitochondria

Muscle samples were obtained from quadriceps muscle of patients submitted to surgery for placement of a hip prosthesis or for femoral fracture. The tissue sample was obtained at the beginning of the surgery and put into a medium at 4°C containing 0.3 M sucrose, 10 mM  $\text{KH}_2\text{PO}_4$  and 1 mg/ml bovine serum albumin (pH 6.50) (medium A). Mitochondria were immediately isolated as described previously (Rustin et al., 1994), mitochondrial protein content was calculated according to Bradford's method (Bradford, 1976), and assays of enzymatic activity were consecutively performed in a DWS (Beckman DU 640, USA) and a SWS (Uvikon 920; Kontron, Switzerland). To convert absorbance to specific activity (expressed in  $\text{nmol min}^{-1} \text{mg protein}^{-1}$ ), we used the equation  $(\Delta A/\Delta t) \times V \times 10^6 / (P_{\text{total}} \times b \times \epsilon)$ , where  $\Delta A$  is the increase in absorbance at 550 nm (absolute value),  $\Delta t$  is the reaction time (min),  $V$  is the volume (in litres) of the cuvette,  $P_{\text{total}}$  is the total amount of mitochondrial protein in the cuvette (mg),  $b$  is the width of the cuvette (cm) and  $\epsilon$  is the extinction coefficient for cytochrome *c* ( $19.1 \text{ mmol}^{-1} \text{ cm}^{-1}$ ) (Berry and Trumpower, 1987).

### 2.2. Cytochrome *c* oxidase assays

All assays were performed at 37°C in 1 ml of medium A.

#### 2.2.1. Double wavelength spectrophotometer assays

We calculated the COX activity from the initial pseudo-linear rate of cytochrome *c* oxidation at 550 nm minus 580 nm. Mitochondrial protein (10  $\mu\text{g}$ ) and reduced cytochrome *c* (10 mM) were added to medium A. The reaction was started by adding 2.5 mM of laurylmaltoside and the cuvette was stirred with a mechanical device (vortex Heidolph-Reax 2000) at maximum speed for 2 s.

#### 2.2.2. Single wavelength spectrophotometer assays

The following studies were consecutively performed in a SWS: (1) mitochondria and 10 mM of reduced cytochrome *c* were added to medium A, and after recording the initial rate (a minimal rate due to membrane integrity), the reaction was started with 2.5 mM of laurylmaltoside; (2) 10 mM of reduced cytochrome *c* and 2.5 mM of laurylmaltoside were added to the medium, and after recording the baseline, the reaction was started with the addition of mitochondria; (3) mitochondria were incubated with 2.5 mM of laurylmaltoside in the medium for 2 min, and, after recording the baseline, the reaction was started with 10 mM of reduced cytochrome *c*. In all cases, the medium in the cuvette was stirred for 2 s using two different methods: a mechanical stirrer or a hand-held plastic rod. Finally, all experiments were performed using different amounts of mitochondria (10, 5, 2 and 1 mg of mitochondrial protein).

A series of five assays with mitochondria from the same sample was performed for each experiment and each spectrophotometer, and five individuals were studied.

### 2.3. Statistical analysis

COX activity of all individuals was calculated with both DWS and SWS, and the results were expressed as mean  $\pm$  standard deviation.

In order to compare the intra-individual variability (or reliability of the method), we calculated the intraclass correlation index (*R*) for each method (DWS and SWS) according to the equation  $R = \sigma_V^2 / (\sigma_V^2 + \sigma_E^2)$ , where  $\sigma_V^2$  is the variance between individuals (inter-individuals) and  $\sigma_E^2$  the variance of estimation error (intra-individuals). Such variances were calculated with a one-way ANOVA using SPSS statistical software.

We also assessed the concordance between both methods (DWS and SWS) by means of intraclass correlation index (*R*). It was calculated as follows:  $R = \sigma_V^2 / (\sigma_V^2 + \sigma_E^2 + \sigma_M^2)$ , where  $\sigma_V^2$  is the variance between

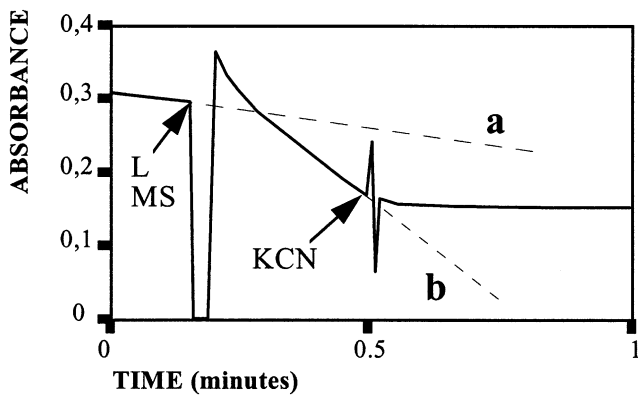


Fig. 1. Spectrophotometric recording of COX activities as measured with a double wavelength spectrophotometer (DWS). (a) Oxidation rate of reduced cytochrome *c* by intact mitochondria; (b) oxidation rate after permeabilization of mitochondria with detergent (laurylmaltoside, 2.5 mM). The reaction is fully sensitive to KCN (200  $\mu$ M). Since DWS avoids the transient scattering by monitoring two wavelengths and recording the difference (which is not possible with SWS), the rate of COX activity is pseudo-linear and easily measurable. L, laurylmaltoside; MS, mechanical stirring.

individuals (inter-individuals),  $\sigma_E^2$  the variance of estimation error (intra-individuals), and  $\sigma_M^2$  the variance between the methods. Such variances were calculated with a two-way ANOVA using SPSS statistical software.

The *R* values were considered as very good if greater than 0.90, and good if between 0.71 and 0.90 (Fermanian, 1984).

### 3. Results and discussion

As a first step in the assay of COX activity using a DWS, we determined the rate of cytochrome *c* oxidation by intact mitochondria in the absence of detergent (Fig. 1, slope a); afterwards, in order to measure the maximal rate of activity, mitochondrial membranes were disrupted by adding a detergent (2.5 mM laurylmaltoside) and vigorous stirring (Fig. 1, slope b). This permitted us to measure both the intactness of the mitochondrial outer membrane and COX activity.

Similar experiments were carried out using a SWS (Fig. 2). The reaction was started with either laurylmaltoside, mitochondria or reduced cytochrome *c*. In all three situations no linear rates for cytochrome *c* oxidation were obtained after mechanical stirring, due to concealment of the true enzyme activity. Since a similar initial trace was also observed even in the presence of KCN, the decrease in the absorbance is most probably a result of changes in the light scattering of the medium, possibly due to physical changes of the mitochondria (swelling due to membrane disruption by laurylmaltoside) or interfering factors such as foam and bubbles (due to the stirring process) rather than to the enzyme activity.

We next reproduced the experiment using hand-driven plastic rods to gently stir and prevent the formation of foam and air bubbles. Under these conditions (Fig. 3A), in the presence of KCN, no significant rate or changes in absorbance of basal trace were observed; conversely, mechanical stirring and the addition of

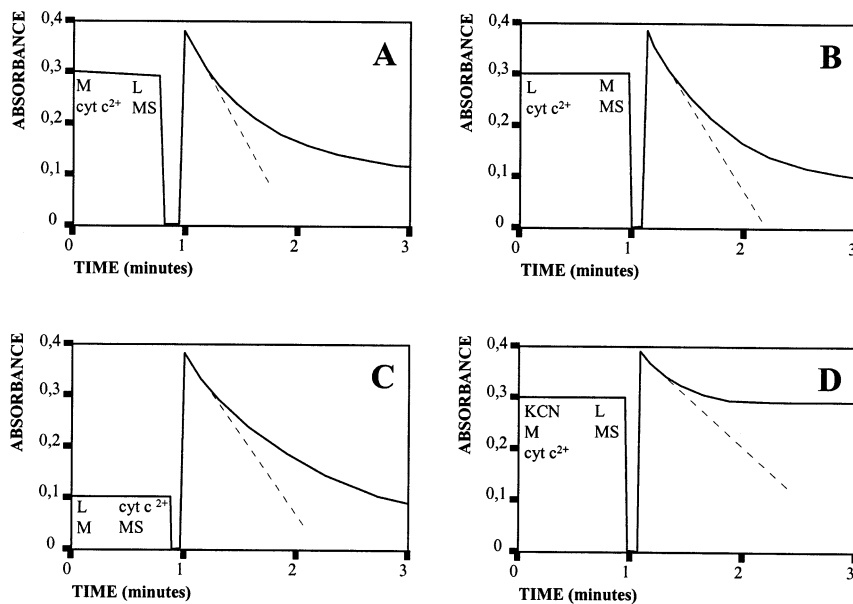


Fig. 2. Spectrophotometric recording of COX activities with a single wavelength spectrophotometer (SWS). Traces correspond to the reaction started with either laurylmaltoside (L, panel A), mitochondria (M, panel B) or reduced cytochrome *c* (cyt  $c^{2+}$ , panel C) and immediate mechanical stirring (MS). In all three conditions, MS causes a large initial absorbance change followed by non-linear rates of cytochrome *c* oxidation. Finally (panel D), under the same conditions as in panel A, but in the presence of KCN (200  $\mu$ M), a similar initial trace (up to 30 s) is also observed after the addition of laurylmaltoside.

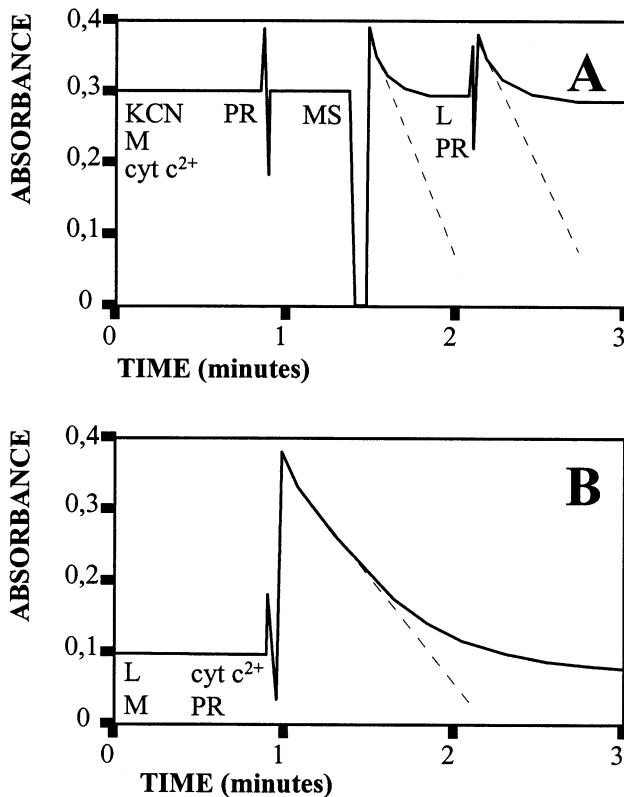


Fig. 3. SWS recording of COX activities. (A) The same mixture containing mitochondria (M), KCN (200  $\mu$ M) and cytochrome *c* (cyt *c*<sup>2+</sup>) in the same cuvette was used throughout the experiment. Manual stirring with a plastic rod (PR) physically blocks the beam and causes a short disruption of the basal trace without any further absorbance change. Mechanical stirring (MS) after removal of the cuvette from the spectrophotometer, and after the addition of laurylmaltoside (L) followed by manual stirring with a plastic rod caused a transient but significant slope in optical absorbance that can be confused with COX activity. (B) Mitochondria were preincubated with laurylmaltoside for 2 min and the assay was started with reduced cytochrome *c*, and stirring with a plastic rod. A pseudo-linear initial rate, which is a reliable estimate of enzyme activity, was obtained.

laurylmaltoside caused a transient but significant slope in optical absorbance which could be related to either bubble formation or to mitochondrial bursting induced by the detergent, respectively. These changes can be easily confused with true cytochrome *c* oxidation. Finally (Fig. 3B), in order to reduce potential interference with the optical changes caused by the detergent addition, we preincubated mitochondria with laurylmaltoside for 2 min, and afterwards added the reduced cytochrome *c*. After gentle stirring with a plastic rod for 2 s, we obtained a pseudo-linear initial rate allowing a confident estimation of the enzyme activity. Under these conditions, rates were found to be proportional to the amount of mitochondrial protein used (Fig. 4).

When we analysed the results of COX assay obtained with the SWS under the optimal conditions (Table 1), we found a low intra-experiment variability

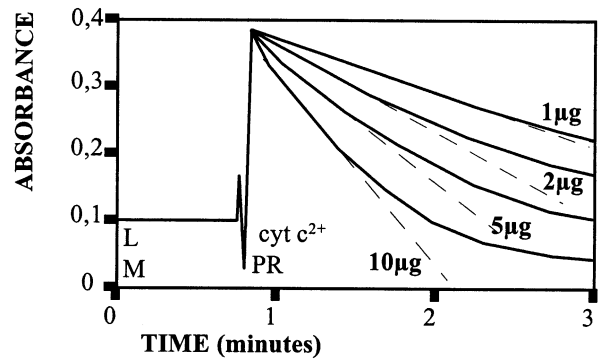


Fig. 4. SWS recording of COX activities using different amounts of mitochondrial protein. Mitochondria (M) were preincubated with laurylmaltoside (L) and the reaction was started with reduced cytochrome *c* (cyt *c*<sup>2+</sup>) stirred with a plastic rod (PR). Use of low amounts of protein (1 or 2 mg) provided longer pseudo-linear rates.

ity ( $R=0.912$ ), which was similar to that obtained using the DWS ( $R=0.957$ ). Additionally, the concordance between the enzyme activities obtained with both methods (DWS and SWS) was also very good ( $R=0.975$ ).

In conclusion, we believe that a reliable estimate of COX activity can be obtained using a SWS if the following simple precautions are taken into account: (1) prevent the formation of foam and air bubbles in the assay mixture using manual stirrers; (2) preincubate the mitochondria with the detergent before the start of the reaction by reduced cytochrome *c* in order to avoid light scattering due to mitochondria swelling and disruption; and (3) use low amounts of mitochondria (1–2 mg of protein) in order to extend the duration of the pseudo-linear initial phase of the COX reaction. Control of such trivial artefacts should be possible using a standard protocol in any professional laboratory using single beam measurement. Since there is an increasing number of laboratories involved in the use of difficult enzymatic screens for respiratory chain diseases, the procedural changes could be important for improving interlaboratory standardization of the COX assay.

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Table 1  
Cytochrome *c* oxidase activities (nmol min<sup>-1</sup> mg protein<sup>-1</sup>) determined with double and single wavelength spectrophotometers

	Individual 1	Individual 2	Individual 3	Individual 4	Individual 5	Total
Double wavelength spectrophotometer						
	2000	1200	508	992	2916	
	1539	952	777	954	3114	
	2203	1289	775	651	2530	
	1986	1205	778	908	2655	
	1874	1121	870	846	2820	
Mean ± S.D.	1920 ± 244	1153 ± 127	742 ± 137	870 ± 134	2807 ± 227	1499 ± 805
Single wavelength spectrophotometer <sup>a</sup>						
	2161	1459	708	772	2338	
	1949	1493	671	784	2970	
	1893	1365	619	627	2107	
	1619	1331	593	637	3118	
	1695	1307	759	711	2119	
Mean ± S.D.	1862 ± 214	1391 ± 81	670 ± 67	706 ± 73	2530 ± 480	1431 ± 755

<sup>a</sup> Mitochondria (1 μg of protein) were preincubated with laurylmaltoside for 2 min. The reaction was started with the addition of reduced cytochrome *c* and the medium was stirred with a plastic rod for 2 s.

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