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Optimal Conditions for Determination of Cytochrome *c* Oxidase Activity in the Rat Heart

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Summary

The determination of cytochrome *c* oxidase (COX) activity represents an important indicator for the evaluation of cell oxidative capacity. However, it has been shown repeatedly that different factors modify the rate of COX activity under various experimental conditions. The most important concern the ionic concentrations of the medium and the application of various detergents for the solubilization of mitochondrial membranes. We found the highest activity of COX in rat heart homogenates and mitochondria at 40-60 mM potassium phosphate. The rate of COX activity is dependent on the detergent/protein (P) ratio. Using n-dodecyl-β-D-maltoside (lauryl maltoside, LM) as the detergent, we obtained the highest activity at LM/P ratios of (50:100):1. By kinetic measurements of low-affinity binding sites in heart mitochondria, we found V_{lim} values of 4.3 and 22.2 μmol cytochrome *c* per min per mg P in the presence or absence of lauryl maltoside, respectively. The K_m values were 16.7 μmol in the presence or absence of lauryl maltoside. Our results thus indicate that 1) the exact assessment of COX activity in heart homogenates and mitochondria requires the determination of optimum phosphate concentrations in the medium used, and 2) even small modifications of the experimental procedure may induce significant differences in the maximum values of COX activity.

Key words

Rat heart • Cytochrome *c* oxidase • Lauryl maltoside • Potassium phosphate

Introduction

The determination of cytochrome *c* oxidase (COX) activity is an important indicator for evaluating the oxidative capacity of cardiomyocytes. COX represents a terminal part of the mitochondrial respiratory chain and its activity thus indicates the maximal capacity for the transfer of reducing equivalents from nutritional substrates to molecular oxygen and its activity may be modified by many factors, such as nucleotides (Bisson *et al.* 1987, Taanman and Capaldi 1993), ions (Buge and Kadenbach 1986, Kirichenko *et al.* 1998) or fatty acids (Thiel and Kadenbach 1989). It is, therefore, not quite clear to what extent the capacity of COX can be utilized in intact cells. Therefore, the exact data on maximum COX activity in heart homogenates and mitochondria as reference values are of importance.

Two different methods are used for the determination of COX activity: (i) polarographic determination of the rate of oxygen consumption (oxygen reduction) (Schneider and Potter 1943, Cooperstein and Lazarow 1951), and (ii) spectrophotometric measurement of the rate of reduced cytochrome *c* oxidation (Cooperstein and Lazarow 1951, Yonetani 1967, Shertzer

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and Cascarano 1972). For the kinetic measurements, the polarographic method has some advantages because the concentration of reduced cytochrome *c* is maintained constant during the estimations by added ascorbate. However, for detection of the maximum rate of COX activity, the spectrophotometric method is preferably used because of its simplicity (Van Kuilenburg *et al.* 1991).

Already in 1951, Cooperstein and Lazarow recommended that optimum ionic concentration should first be determined for particular tissue preparations and especially when different sources of cytochrome *c* are used. For various cell preparations, the concentrations of potassium phosphate in the medium were recommended to range from 10 to 100 mM (Schneider and Potter 1943, Wharton and Tzagoloff 1967, Yonetani 1967, Zimmermann and Kadenbach 1992) and various values of COX activity were obtained.

Another important factor activating this enzyme appears to be the concentration of detergent used for solubilization of the mitochondrial membrane. Lauryl maltoside (LM) has been recommended because it supports a high turnover number of the enzyme (Robinson and Capaldi 1977, Mahaparto and Robinson 1990). A two- to threefold increase of COX activity was found in homogenates or mitochondria treated with 0.1 % lauryl maltoside (Kadenbach *et al.* 1987). Various protocols have been used for lauryl maltoside treatment of mitochondria or homogenates. This could also modify the requirements for the optimum concentration of potassium phosphate in the medium.

In the literature, we can find various methods for the determination of COX activity with different results, according to the experimental conditions. Therefore, the aim of our study was focused on the improvement and the standardization of the measurements of COX activity in the rat heart.

Methods

All experiments using laboratory animals were performed according to the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH publication No 85-223, revised 1985).

Male Wistar rats aged 90 days were used. The animals were killed by decapitation under light narcosis. The heart was excised and washed in 0.9 % NaCl and the left and right ventricles were separated (Fulton *et al.* 1952). Heart tissue samples (50 mg of heart tissue/ml) were cut by scissors and homogenized at 0 °C for 15 s by ultrathurax and then by a teflon-glass homogenizer in 25 mM sucrose (Sigma, USA), 10 mM Tris-HCl (Tris-hydroxymethyl aminomethane, Sigma), 1 mM EDTA (Na_2 salt, Serva Feinbiochemica, Heildelberg), pH 7.4. The homogenate was centrifuged for 10 min at 600 *g* and the resulting supernatant for 10 min at 10 000 *g*. The mitochondrial sediment was washed with an isolation medium and suspended in the same solution. The homogenates and isolated mitochondria were stored at –20 °C.

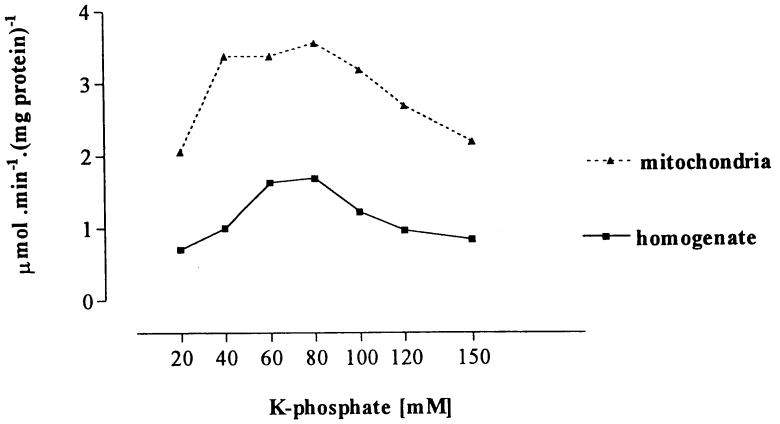

Fig. 1. *Effect of potassium phosphate concentrations on COX activity of rat heart homogenates and mitochondria.*

The activity of COX in the homogenate and mitochondria was determined spectrophotometrically by measuring the rate of cytochrome *c* oxidation at 550 nm and at 25 °C (Wharton and Tzagaloff 1967). Bovine cytochrome *c*

(Sigma C 3006) was reduced with sodium hydrosulfite (Sigma, USA) followed by the removal of excess sodium hydrosulfite on a Sephadex G-25 column (Pharmacia, Fine Chemicals AB, Uppsala, Sweden). Phosphate concentrations (p.a., Lachema Brno, Czech Republic), lauryl maltoside concentrations (n-dodecyl-β-D-maltoside, Sigma, USA) and LM/P ratios are shown in the Figures. Enzyme activities were expressed as micromoles of cytochrome *c* oxidized per min per mg protein. Proteins were determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

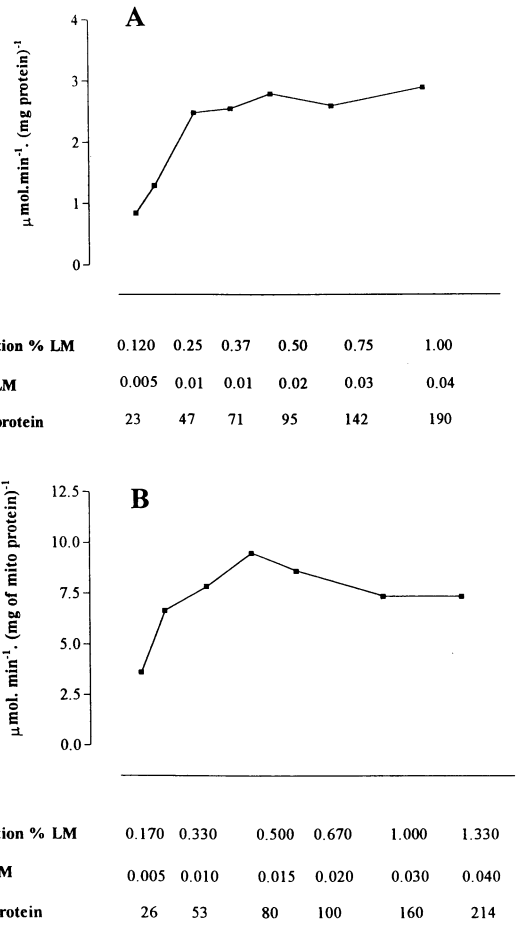

Fig. 2. *Activation of COX activity by increased LM/protein ratio in rat heart homogenates* ***[A]*** *and in rat heart mitochondria* ***[B]****. LM – lauryl maltoside.*

Results and Discussion

As indicated in Figure 1, we found maximum COX activity in rat heart homogenate at 60-80 mM potassium phosphate and in the heart mitochondria at 40-80 mM potassium phosphate. At 20 mM phosphate, COX activity of homogenates and mitochondria represented 41 % and 55 % of maximum values, respectively. At 100–150 mM potassium phosphate, the rates of cytochrome *c* oxidation were also lower, i.e. 47 % and 75 % of maximum values. At 40–60 mM potassium phosphate and at the LM/P ratio 5 and 25, we observed maximum COX activity values of 1.6 and 3.6 μmol of cytochrome *c* oxidized per min per mg protein in the rat heart homogenates and mitochondria, respectively (Fig. 1). These values are in agreement with earlier reported data (Gold and Costello 1974, Ito *et al.* 1989, Kalous *et al.* 1989). In these experiments, samples of homogenates (5 mg/ml) and mitochondria (2 mg/ml) were treated at 0 °C for 2-3 min with lauryl maltoside (final concentration 1 %), LM/P was about 5 and 25 for the homogenates and mitochondria, respectively. For determination of COX activity, detergent-treated samples were diluted 10–20 times.

In further experiments, we modified the procedure of LM treatment. We incubated small aliquots (10–20 μl of 50 times diluted homogenates and 20 times diluted mitochondria) with lauryl maltoside directly in the cuvette (the final concentration being 1 %). Under these conditions, there was a tenfold increase of the LM/P ratio to a value of about 200. As shown in Figures 2a and 2b, COX activity of the homogenates increased to about 3 μmol cytochrome *c* per min per mg protein and for mitochondria to about 10 μmol, which are the values 2-3 times higher than those presented in Figure 1. It is evident that COX activity decreased when the LM/P ratio was below 50 (Figs 2a and 2b). Maximum values of COX activity for both homogenates and mitochondria could thus be obtained at the LM/P ratio of about 100.

Under the conditions of a high LM/P ratio, we have repeatedly tested the effect of various potassium phosphate concentrations (20–120 mM) on COX activity. As shown in Figure 3, the optimum concentration of potassium phosphate (60 mM) was the same as in the previous experiments. However, in comparison with the measurements at the lower LM/P ratio, the peak of COX activity was sharper due to the steeper decrease at lower and higher phosphate concentrations. Activation of COX activity by a high LM/P ratio could be due to the release of some subunits of the enzyme complex. Such an activating effect was described by Weishaupt and Kadenbach (1992) on isolated COX from beef hearts after selective removal of the VIb subunit.

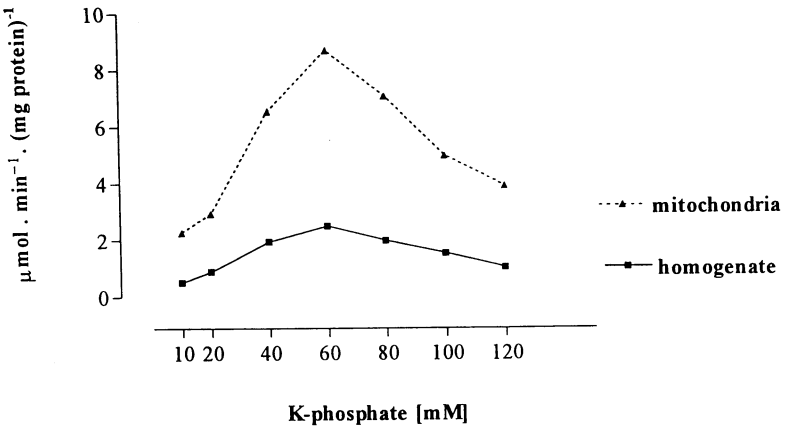

Fig. 3. *Effect of potassium phosphate concentration on COX activity of rat heart homogenates and mitochondria at the optimum LM/protein ratio. LM – lauryl maltoside.*

We evaluated the activating effect of lauryl maltoside under optimum conditions (30 μM cytochrome *c*, 60 mM potassium phosphate, LM/P ratio 100) by comparison with values obtained without lauryl maltoside. In isolated mitochondria, there was a twofold increase of COX activity at the LM/P ratio of 38 and a fourfold increase at the LM/P ratio of 152 (data not shown).

Under these experimental conditions (60 mM potassium phosphate, LM/P ratio 80-150) we measured COX activity in homogenates and mitochondria of fourteen 90-day-old male rats and we obtained reference values for the homogenates and for mitochondria isolated from the left and right ventricles, respectively (Table 1).

Table 1. Specific activity of COX in heart homogenates and mitochondria.

COX activity	n	Right ventricle	Left ventricle
Homogenates	14	2.9±0.1	3.1±0.2
Mitochondria	14	14.8±0.9	16.0±0.5

Data (μmol cytochrome c / min / mg protein) are means ± S.E.M. Right and left ventricles were isolated from 90-day-old male rats, n indicates the number of animals.

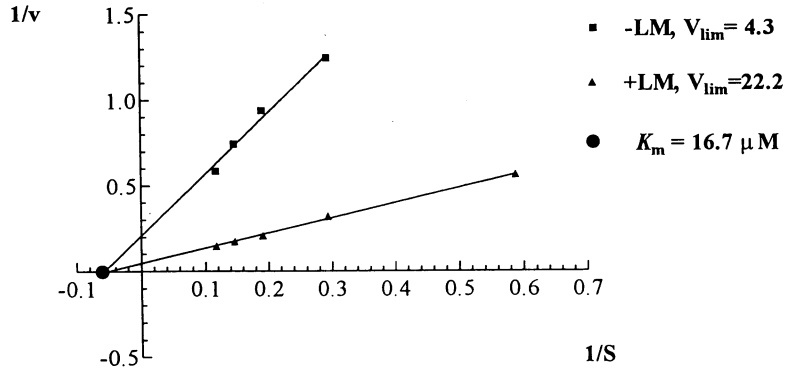

Fig. 4. *Effect of lauryl maltoside (LM) on kinetic properties of COX of heart muscle mitochondria.*

By kinetic measurements of low-affinity binding sites, we obtained reference values for V_{lim} and for K_m of COX activity in the presence or absence of the lauryl maltoside for heart homogenates and mitochondria (Fig. 4). The data indicate that the lauryl maltoside highly increases V_{lim} but does not affect the K_m values.

On the basis of these results we can summarize that by increasing the detergent to protein ratio above values reported in the literature we may further enhance the activity of COX. This high enzyme activity is an important reference value providing information about the oxidative capacity and the amount of the enzyme in particular cells.

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Reprint requests

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