

# Spectroscopic Determination of Cytochrome c Oxidase Content in Tissues Containing Myoglobin or Hemoglobin

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A simple spectroscopic method for determining the cytochrome c oxidase, cytochrome a, a<sub>3</sub>, content in tissue and mitochondria samples independent of myoglobin or blood contamination is described. Using tissue homogenates solubilized in Triton X-100, this assay relies on the selective reduction of mitochondrial cytochromes by the action of potassium cyanide. Monitoring the optical absorbance of these samples at 605 nm provided a quantitative determination of cytochrome c oxidase content in the presence of myoglobin or blood. The cytochrome c oxidase content of porcine heart mitochondria and whole tissue was determined to be 0.85 nmol/mg protein and 30.5 nmol/g wet wt, respectively. © 1996 Academic Press, Inc.

The determination of cytochrome c oxidase, or cytochrome a, a<sub>3</sub>, in biological tissues is a useful marker of mitochondrial content and oxidative metabolism adaptations (1–3). There are two approaches for determining cytochrome oxidase in tissues. One approach is to determine the enzymatic activity via reduced cytochrome c stimulation of oxygen consumption (4, 5) or cytochrome c oxidation directly (5) in specially prepared tissue homogenates. Another method relies on quantitative spectrophotometric determination of cytochrome c oxidase content in nonionic detergent-treated homogenates (6) or complex treatments of crude tissue homogenates (14). The quantitative spectrophotometric method is the only procedure which provides an absolute concentration of cytochrome oxidase. In addition, this absolute spectrophotometric determination can be performed without complications associated with proper tissue fractionation and modification of enzymatic activity.

A quantitative spectrophotometric approach for determining mitochondrial cytochromes in tissue samples was described by Williams (6). Using this approach, homogenized tissue is dissolved into a 2%

Triton X-100 solution to eliminate light scattering effects. The differential absorbance at 605 and 630 nm during chemically induced maximum oxidation and reduction of the sample was used to determine the absolute concentration of cytochrome oxidase content. The oxidation is usually performed with a strong oxidizing agent, such as ferricyanide, while the reduction is accomplished with ascorbate in combination with sodium hydrosulfite. This approach has been successfully used in numerous mitochondrial preparations (2) as well as tissues including liver (6) and kidney (1). However, we have found that in tissues containing myoglobin, such as heart or skeletal muscle, this approach overestimates the cytochrome c oxidase content due to the contribution of myoglobin to the oxidation–reduction difference spectrum. Hemoglobin from blood contamination during tissue isolation or biopsy procedures can lead to similar errors.

The purpose of this study was to develop a method of determining cytochrome c oxidase content in tissue homogenates in the presence of myoglobin or hemoglobin. We have found that the selective spectrophotometric effects of cyanide on cytochrome c oxidase in detergent-treated homogenates provide a simple and accurate assay for cytochrome c oxidase content.

## MATERIALS AND METHODS

Pig hearts were collected from chloralose (100 mg/kg intravenously)-anesthetized animals after a midline thoracotomy. The animals were heparinized (10,000 units intravenously) prior to harvesting the heart. The heart was then immediately perfused via the aorta in a retrograde fashion with 1 liter of isolation medium (0.3 M sucrose, 10 mM Hepes, 0.2 mM K<sub>2</sub>EDTA, pH 7.2) at 5°C. The left ventricle was dissected free from fat, large vessels, and the right ventricle free wall. Approximately 5 g of heart muscle was taken for tissue homogenization, while the remaining myocardium was used

for mitochondria isolation. This procedure permitted the direct comparison of heart tissue and mitochondria collected from the same animal.

The tissue homogenization was performed by placing a sample of heart muscle (~5 g) in an equal weight of 0.1 M sodium phosphate buffer at pH 7.0. The tissue was then minced with scissors at 5°C. This mixture was then homogenized using two 5-s treatments at maximum speeds with a tissue homogenizer (Virtis, Gardiner, NY) at 5°C. Excessive treatment in the homogenizer or permitting high temperatures during homogenization resulted in the generation of oxidized myoglobin, which should be avoided (see Results). Approximately 1-ml samples of the homogenate were weighed and then dissolved in 5 ml of 2% (v/v) Triton X-100 in 0.1 M sodium phosphate buffer (pH 7.0). After mixing, the suspension was centrifuged to remove any residual connective tissue and solid material. The resulting pellet was white with no evidence of unlysed material. The solid material composed of less than 3% of the total volume based on wet weight-dry weight ratios. No correction for this fractional volume was made in the calculations. This supernatant was then directly used in the spectrophotometric cytochrome oxidase assays discussed below. Protein analysis revealed a concentration of  $140 \pm 20$  mg protein/g wet weight in these homogenates. The wet weight to dry wet ratio was  $4.7 \pm 0.3$ .

Heart mitochondria were prepared as previously described (7). The final mitochondrial incubation medium was 130 mM KCl, 20 mM Hepes, 2.5 mM MgCl<sub>2</sub>, 0.5 mM K<sub>2</sub>EDTA, pH 7.2. Respiratory rates were determined at 37°C in a sealed glass chamber with a Clark oxygen electrode (Yellow Springs, Yellow Springs, OH) using a solubility of O<sub>2</sub> of 199 nmol O<sub>2</sub>/ml. The respiratory quotient (State 3/State 4) ranged from 8 to 9. For the cytochrome oxidase assay, 11 to 18 mg of mitochondrial protein (1 ml of mitochondria stock solution) was dissolved in 2 or 3 ml of 2% Triton X-100 in 0.1 M phosphate buffer at pH 7.0. No centrifugation was necessary in these samples since no solid matter was observed. These samples were then directly used in the spectrophotometric assay for cytochrome oxidase. Protein was determined by the biuret method (8).

One milliliter of Triton X-100-solubilized tissue or mitochondria was placed in two spectrophotometric cuvettes. These were then placed in a spectrophotometer (Perkin Elmer, Norwalk, CT) and all measurements were made as the optical absorbance differences between the two cuvettes. Typically, chemical modifications were only made to one cuvette (experimental cuvette) with the other serving as a reference. Difference spectra were recorded on a strip chart recorder as well as digitized using an A/D converter (Strawberry Tree, Sunnyvale, CA) interfaced with a computer (Apple Computers, Cupertino, CA). The OD was calculated

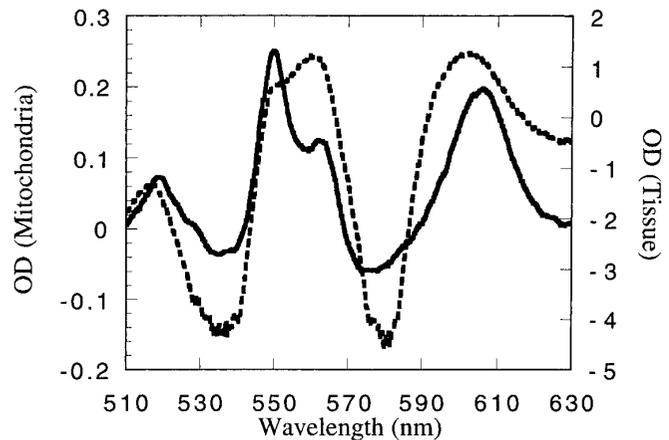


FIG. 1. Effect of sodium hydrosulfite on the optical absorbance of heart tissue (broken line) and mitochondria (solid line) Triton X-100-treated preparations. All spectra presented are difference spectra of the reference cuvette versus the experimental cuvette. Difference spectra were collected versus untreated control cuvette with appropriate water additions to keep tissue concentration constant. Heart tissue sample contained 0.063 g wet wt/ml. Mitochondria contained 4.65 mg protein/ml. OD is the optical density difference between the two cuvettes in this and all following figures.

from the difference in absorbance at 630 versus 605 nm or in samples with a drifting baseline the difference from a baseline drawn between 630 and 575 nm (see (9)). All data presented in figures show the optical difference (OD) between the reference and experimental cuvette.

## RESULTS

In whole tissue homogenates, addition of sodium hydrosulfite to one of the cuvettes resulted in a difference spectrum which was characteristic of a combination of both cytochromes and myoglobin. This is most apparent when a tissue sample spectrum is compared directly with a mitochondrial preparation which does not contain myoglobin, as shown in Fig. 1. Since myoglobin contributes significantly to the absorbance at 605 nm, the presence of myoglobin results in the overestimation of the cytochrome oxidase contribution when the standard 605–630 wavelength pair is used.

Since cytochrome oxidase is active in nonionic detergents (see Ref. 4), we reasoned that the addition of potassium cyanide should prevent the oxidation of cytochrome by oxygen and result in a selective reduction of cytochrome c oxidase which could be observed at 605 nm. Low concentrations of cyanide do not interact significantly with ferric hemoglobin or myoglobin (10). Consistent with this notion, oxygen consumption in Triton X-100-treated homogenates was stimulated (~300%) by sodium ascorbate (15 mM) and inhibited (~50%) by cyanide (2 mM) in the presence of ascorbate. As shown in Fig. 2, potassium cyanide (2 mM) added

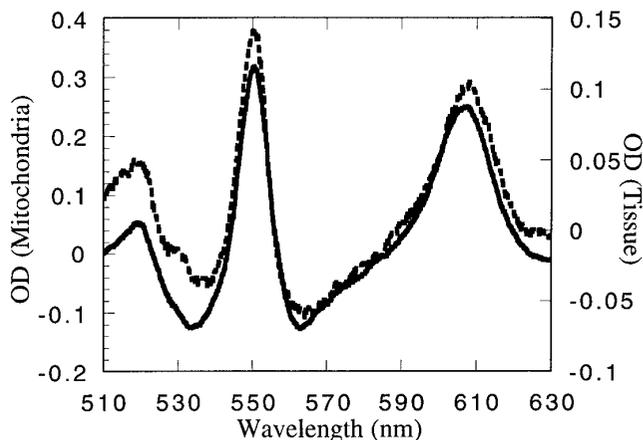


FIG. 2. Effect of 2 mM potassium cyanide on the optical properties of heart tissue (broken line) and mitochondria (solid line) preparations. Difference spectra were collected versus untreated control cuvette with appropriate water additions to keep the tissue concentration constant. The mitochondria concentration was 4.65 mg protein/ml. The heart tissue concentration was 0.070 g wet wt/ml.

to the Triton X-100-solubilized tissue or mitochondria resulted in identical spectral characteristics consistent with a cytochrome c and cytochrome c oxidase reduction-oxidation difference spectrum. Absorption maxima were observed at 605 nm (cytochrome c oxidase), 550 nm (cytochrome c), and 520 nm (combinations of both cytochromes). The reduction of cytochrome c oxidase was complete in 5 min at 24°C. These data suggest that cytochrome c was being oxidized by cytochrome c oxidase in Triton X-100 and that cyanide blocked this process. Over a long period of time (10 to 15 min), an absorbance at 563 nm, consistent with cytochrome b, was also observed. This suggests that even the oxidation of cytochrome b was still linked to cytochrome c oxidase in these preparations.

The reducing equivalent dependence of the cyanide effect was confirmed by adding 50  $\mu$ l of 0.05 M potassium ferricyanide to both cuvettes before the addition of cyanide to completely oxidize the environment. Under these oxidizing conditions, only weak absorbance bands in the 548- and 584-nm regions were observed consistent with a spin state transition in the ferric heme of cytochrome, as described by Wilson *et al.* (11) under similar conditions in intact mitochondria. The need for reducing equivalents in this process was further confirmed by the addition of 15 mM sodium ascorbate which slightly reduced cytochrome c oxidase under control conditions and increased the rate of cytochrome reduction in the presence of cyanide (not shown). However, ascorbate did not increase the maximum absorbance at 605 nm in the presence of cyanide in either mitochondria or tissue preparations nor did ascorbate alter the spectral characteristics.

The effect of cyanide on mitochondria preparations

was directly compared to the standard sodium hydrosulfite reduction procedure to determine the extent of cytochrome reduction by cyanide. In Fig. 3 difference spectra collected with cyanide or sodium hydrosulfite are shown. Except for the cytochrome b absorbance bands observed at 563 and 530 nm, the cyanide and hydrosulfite spectra were nearly identical in terms of spectral shape and absorbance magnitude. The cyanide treatment resulted in a slightly lower (10%) absorbance at 605 nm when compared to sodium hydrosulfite as observed by Van Buuren *et al.* (12). These investigators demonstrated that the oxygenated-deoxygenated extinction coefficient of cyanocytochrome oxidase is 10% lower than the native enzyme. This is likely due to the contribution of cytochrome  $a_3$  in the sodium hydrosulfite spectrum and not in the cyanide spectrum since it

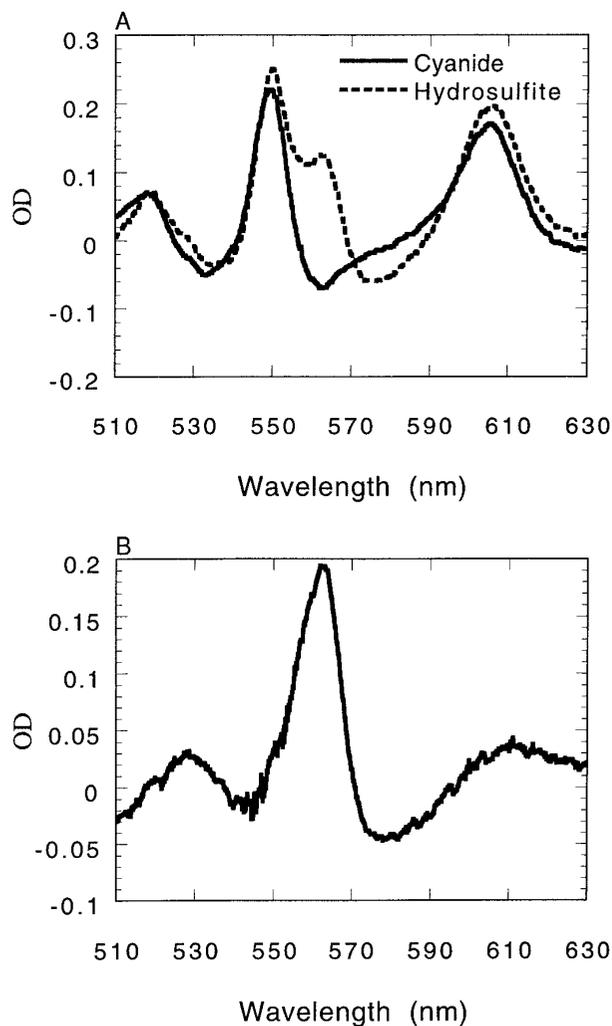


FIG. 3. Effect of potassium cyanide and sodium hydrosulfite on the absorption spectra of solubilized mitochondria. Mitochondria concentration was 4.65 mg protein/ml. (A) Difference spectrum was obtained with 2 mM cyanide or sodium hydrosulfite. (B) Difference spectrum of the data presented in A.

TABLE 1  
Cytochrome Oxidase Content in Porcine Heart Tissue and Mitochondria

Sample	Reducing agent	Cytochrome oxidase content
Mitochondria	Cyanide ( $n = 12$ )	$0.84 \pm 0.058$ nmol/mg protein
Mitochondria	Hydrosulfite ( $n = 6$ )	$0.85 \pm 0.055$ nmol/mg protein
Heart tissue	Cyanide ( $n = 17$ )	$30.5 \pm 1.6$ nmol/g wet wt

*Note.* The millimolar extinction coefficient for cytochrome oxidase varies in the literature from  $26 \text{ OD}^{-1} \text{ cm}^{-1}$  (15) to  $12 \text{ OD}^{-1} \text{ cm}^{-1}$  (6). The millimolar extinction coefficients used for the difference spectra in this table were  $12 \text{ OD}^{-1} \text{ cm}^{-1}$  for sodium hydrosulfite and  $10.8 \text{ OD}^{-1} \text{ cm}^{-1}$  for potassium cyanide.  $\pm$  SD.

complexes with cyanide. This conclusion was supported by the observation that sodium hydrosulfite, added after cyanide, did not result in a 10% increase in 605 absorbance due to the high retention of cyanide to cytochrome  $a_3$  in the presence of excess cyanide (12). From these data, we assumed that cyanide was as effective in reducing cytochrome oxidase for quantitative spectroscopic techniques as long as this 10% correction was made. Quantitative data, using a corrected millimolar extinction coefficient for the cyanide data, are presented in Table 1. Serial dilution studies revealed a linear relationship between mitochondria and tissue content with the 605 absorbance over a 10-fold range of concentrations.

In previous studies (1, 6), sodium ferricyanide was added to the reference cuvette to assure complete oxidation of the cytochromes. This is extremely problematic in the presence of myoglobin since ferricyanide would also generate oxidized (i.e., ferric) myoglobin resulting in gross absorbance changes in the 600 nm region (Fig. 4). However, we found that the use of ferricyanide was not necessary for cytochrome oxidase since it is apparently maximally oxidized under normal isolation conditions (13). To confirm this, we found that the addition of sodium ferricyanide to the reference cuvette of a mitochondrial preparation, without myoglobin, had no effect on the absorption spectrum in the 600 nm region (Fig. 5). Cytochrome b was apparently partially

reduced in this preparation since an absorbance at 563 was observed with ferricyanide.

It was expected that myoglobin and hemoglobin would behave similarly with regard to cyanide and blood contamination and would not interfere with using cyanide as an "effective" cytochrome oxidase reducing agent (10). To test this hypothesis, pig blood was added to the mitochondrial preparation and the assay was performed using cyanide and sodium hydrosulfite. The addition of hemoglobin had no effect on the spectral characteristics or the quantitative determination of cytochrome oxidase with cyanide. However, the hemoglobin contamination greatly distorted the sodium hydrosulfite data (Fig. 6).

## DISCUSSION

We have shown that the addition of cyanide to Triton X-100-solubilized mitochondria or tissue permits a quantitative spectroscopic determination of cytochrome c oxidase independent of myoglobin or blood contamination. This approach takes advantage of the ongoing cytochrome c oxidase reaction occurring in the

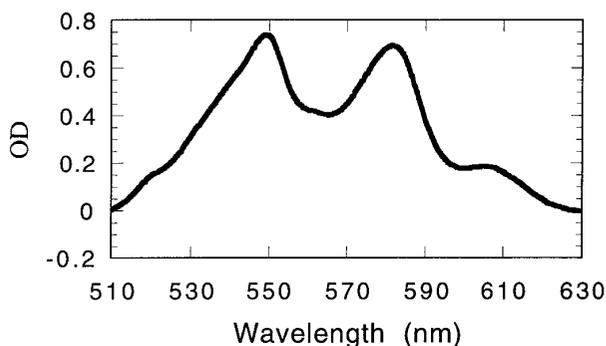


FIG. 4. Effect of ferricyanide (2.5 mM) on the absorbance spectrum of solubilized heart tissue. The heart tissue concentration was 0.063 g wet wt/ml.

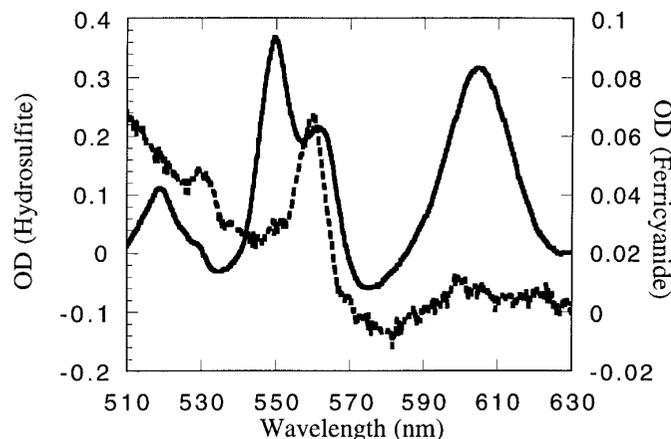


FIG. 5. Effect of ferricyanide (broken line; 2.5 mM) on the absorbance spectrum of solubilized mitochondria. A sodium hydrosulfite spectrum (solid line) is also presented as a reference. The mitochondria concentration was 4.65 mg protein/ml.

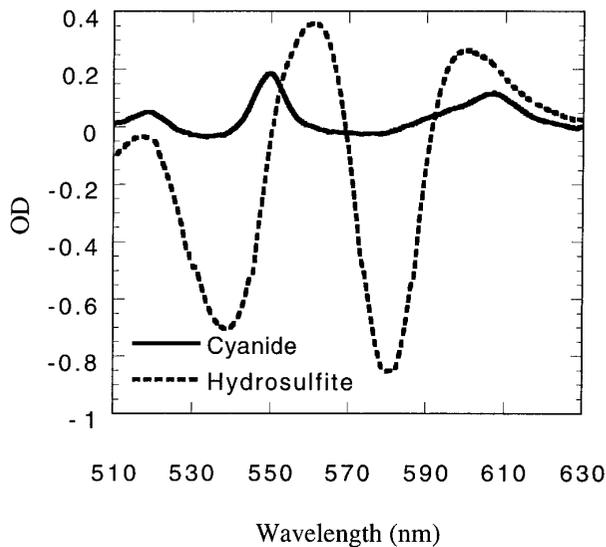


FIG. 6. Comparison of potassium cyanide and sodium hydrosulfite as reducing agents in heart mitochondria contaminated with  $0.5 \mu\text{g/ml}$  pig hemoglobin. The mitochondria concentration was  $4.5 \text{ mg protein/ml}$ .

Triton X-100-solubilized preparation. This reaction utilizes reducing equivalents carried over in the solubilization process from tissue/mitochondria or with added exogenous sources. When this reaction is inhibited with cyanide, a selective and complete reduction of cytochrome c and cytochrome c oxidase is observed. External reducing equivalents, such as ascorbate, were not found to be necessary for the reduction of cytochrome oxidase in this preparation, but ascorbate did increase the rate of reduction. To standardize conditions, and avoid time waiting for equilibration, we recommend the use of  $10 \text{ mM}$  ascorbate in these assays. Cytochrome c oxidase was found to be nearly completely oxidized under control conditions eliminating the need of an external oxidizing agent, such as ferricyanide, to generate the oxidized cytochrome reference.

The simple and rapid aspects of this assay make it also feasible as a replacement to protein measurements for normalizing mitochondria content for *in vitro* studies. Either sodium hydrosulfite or cyanide could be used as the reducing agent with appropriate extinction coefficient. Since standard curves and incubation times are

required in most protein evaluations, this simple spectrophotometric technique can provide a much more rapid measure of mitochondria concentration in a single measurement.

In summary, a spectroscopic method is presented which provides a simple and rapid determination of cytochrome oxidase content in tissue samples with a high concentration of myoglobin or in blood-contaminated tissue biopsy samples.

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