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Optical spectroscopy in turbid media using an integrating sphere: Mitochondrial chromophore analysis during metabolic transitions

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ABSTRACT

Recent evidence suggests that the activity of mitochondrial oxidative phosphorylation complexes (MOPCs) is modulated at multiple sites. Here, a method of optically monitoring electron distribution within and between MOPCs is described using a center-mounted sample in an integrating sphere (to minimize scattering effects) with a rapid-scanning spectrometer. The redox-sensitive MOPC absorbances (~465–630 nm) were modeled using linear least squares analysis with individual chromophore spectra. Classical mitochondrial activity transitions (e.g., ADP-induced increase in oxygen consumption) were used to characterize this approach. Most notable in these studies was the observation that intermediates of the catalytic cycle of cytochrome oxidase are dynamically modulated with metabolic state. The MOPC redox state, along with measurements of oxygen consumption and mitochondrial membrane potential, was used to evaluate the conductances of different sections of the electron transport chain. This analysis then was applied to mitochondria isolated from rabbit hearts subjected to ischemia/reperfusion (I/R). Surprisingly, I/R resulted in an inhibition of all measured MOPC conductances, suggesting a coordinated down-regulation of mitochondrial activity with this well-established cardiac perturbation.

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Over the past several years, there has been increased interest in measuring the enzymatic activity of mitochondrial oxidative phosphorylation complexes (MOPCs).[1] This has been stimulated by the observation of numerous MOPC posttranslational modifications [1–5], increased detection of human diseases associated with mitochondrial defects [6], and attempts to create a systems approach to the understanding of mitochondrial function [7]. Methods to monitor MOPC activity include intact mitochondrial studies with inhibitors and artificial substrates, assays on extracted protein in solution [8], and assays on extracted intact protein complexes via blue native electrophoresis [9]. However, these approaches are limited by altered redox states, absent or modified mitochondrial membrane potential ($\Delta\Psi$), unphysiological substrate availability, and only partial MOPC reactions detected with no $\Delta\Psi$ and altered protein conformations in native gels. Thus, a method of simultaneously monitoring MOPC activity under normal redox and substrate conditions in intact mitochondria may provide more relevant information.

It has been known since the pioneering studies of Chance and Williams [10–13] and Halestrap [14,15] on isolated mitochondria that optical spectroscopy can be used to monitor the distribution of reducing equivalents within and between MOPCs [16–19]. These original studies employed custom-built, split-beam spectrophotometers with large photomultiplier tubes positioned near the experimental sample to compensate for the effects of light scattering. Although technically and experimentally innovative, these systems required high concentrations of mitochondrial protein (up to 12 mg/ml, perhaps outside the linear range of detection) to achieve adequate signal-to-noise, necessitating the performance of respiration experiments at room temperature and often with hydrogen peroxide (up to 5 mM) to maintain sufficient oxygenation. Additional limitations of these early studies included limited spectral bandwidth and slow spectrometer scanning rates. The Wittenbergs later provided improvements to mitochondrial spectroscopy in their assessment of myoglobin-mediated oxygen delivery to isolated cardiac myocytes [20,21]. In particular, they expended spectral acquisition to include the highly scattering Soret frequencies and obtained reasonable signal-to-noise using lower protein concentrations (~4–5 mg/ml). However, their studies focused primarily on mitochondrial oxygen limitation under conditions of myoglobin desaturation and did not directly examine MOPC redox transitions or driving forces for electron transport.

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[1] *Abbreviations used:* MOPC, mitochondrial oxidative phosphorylation complex; SR, substrate reduction, ATPs, ATP synthesis; CR, chemical reduction; I/R, ischemia/reperfusion; UV, ultraviolet; P_i, inorganic phosphate; TPP^+, tetraphenylphosphonium; FAD, flavin adenine nucleotide; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; PCA, principal component analysis.

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The purpose of the current study was to adapt the original methods of Chance and Williams, Halestrap, and the Wittenbergs to monitor the mitochondrial cytochrome redox state using rapid-scanning spectroscopy for simultaneous chromophore analysis together with a center-mounted integrating sphere system to minimize the effects of light scattering. This approach permitted the simultaneous collection of absorption data over a large spectral bandwidth (350–745 nm), providing a redundant analysis of mitochondrial chromophore states using the alpha, beta, and Soret bands. The complex spectral data obtained were analyzed using reference spectra obtained from purified chromophore spectra. Importantly, our method permitted the use of low protein concentration (1 mg/ml), physiological temperature regulation and substrate availability, maintenance of respiratory medium oxygenation without the use of hydrogen peroxide, high maximum respiratory rates and respiratory control ratios, and rapid spectral acquisition rates (~100 ms). Furthermore, using Onsager relationships of flux (oxygen consumption) versus driving forces (chromophore redox states, $\Delta\Psi$) [22], we calculated the effective conductance of oxidative phosphorylation, analogous to conductance measurements of an electrical circuit.

Using this approach, we performed a comprehensive examination of the mitochondrial chromophore responses to carbon substrate reduction (SR) and ATP synthesis (ATPs) transitions as well as chemical reduction (CR) to validate the methodology. These studies demonstrated the ability to fit the redox-sensitive optical spectral changes associated with these transitions to known optical properties of MOPC chromophores. Analysis of these data using linear least squares fitting and unbiased principal component analysis revealed novel distributions of reducing equivalents within ubiquinone/cytochrome *c* oxidoreductase (complex III) and cytochrome oxidase (complex IV), especially in the transition from SR to ATPs. This approach then was applied to mitochondria isolated from perfused rabbit hearts following ischemia/reperfusion (I/R) to examine which MOPC activities were affected by this well-established pathophysiological challenge of energy metabolism.

Materials and methods

Integrating sphere and system design

All optical measurements were made in a 6-inch-diameter integrating sphere with Spectraflect reflectance coating (model RTC-060-SF, LabSphere, North Sutton, NH, USA). A custom-made, cylindrical, water-jacketed quartz cuvette (12 mm i.d., 20 mm o.d., 4 ml total volume, Chemglass Life Sciences, Vineland, NJ, USA) was positioned in the sphere using a center-mounted sample holder (model CSMH-RTC-CUV-SF, LabSphere). A cylindrical geometry was selected to improve the mixing characteristics of the system. For these studies, the chamber was filled to a maximum volume of 2 ml, leaving 2 ml of headspace. The fixed geometry of the sphere and the center-mounted sample holder made sealing of the chamber for oxygen consumption measures difficult. In addition, conventional oxygen electrodes differentially absorbed light, thereby decreasing the bandwidth of our measurements. As such, to obtain accurate measurements of mitochondrial oxygen consumption, all respiration experiments were performed in parallel in sealed glass chambers as detailed below. Lines for the water jacket were introduced using accessory ports around the sphere's diameter and were covered with black foil to eliminate ambient light. A remote stirring device (Variomag Mini, Variomag-USA Electron Stirrers, Daytona Beach, FL, USA) was placed beneath the center-mounted chamber and connected to an external control unit set at 1200 rpm. White light was generated at 500 W using a CrimeScope (model CS-16-500, Jobin Yvon, Spex Forensics Division, Edison, NJ, USA). After passing through a focusing assembly, incident light was delivered to the integrating sphere via a 1000-μm-diameter optic fiber (Ocean Optics, Dunedin, FL, USA). The terminal end of the fiber was attached to the sphere on a side port equipped with a collimating lens (74-UV, Ocean Optics). The small bore of the optical fiber did not deliver the entire output of the light source. However, to monitor the effects of the light, two experiments were conducted. The first experiment measured the temperature of the mixing chamber with mitochondria (1 nmol cytochrome aa_3/ml) while the light was turned off and on for 5-min intervals. No temperature change was detected within the resolution of the device (0.1 °C). In the second experiment, mitochondria were placed in the chamber under normal experimental conditions (~5 min) with and without the light being impinged on the chamber. The mitochondria were rapidly removed from the chamber, and the state 2 and state 3 respiratory rates were determined. In three paired experiments, no difference in state 2 or state 3 respiratory rates was determined in the presence or absence of the illumination. This is consistent with the previous observations of Joubert and coworkers [23] using high-power ultraviolet (UV) pulses on a similar preparation. A second 1000-μm-diameter fiber was connected to the detector port at the bottom of the sphere and attached to a rapid-scanning spectrometer (model QE-65000, Ocean Optics). Spectra were collected at 100 ms integration using SpectraSuite software (Ocean Optics) installed on a personal laptop computer.

Mitochondrial isolation and respiration

All animal experimentation was approved by the National Heart, Lung, and Blood Institute animal care and use committee. Left ventricular mitochondria were isolated from in situ perfused porcine hearts as described previously [24] using buffer containing 0.3 M sucrose, 10 mM Hepes, and 0.2 mM K_2EDTA (pH 7.1). The standard experimental buffer used for mitochondrial respiration contained 125 mM KCl, 15 mM NaCl, 20 mM K-Hepes, 1 mM KEGTA, 1 mM K_2EDTA, and 5 mM $MgCl_2$ (pH 7.1) at 37 °C. Sphere studies were conducted on mitochondrial concentrations of 1 to 2 nmol of cytochrome aa_3/ml, which were found to remain in the linear domain of the system (see below). Cytochrome aa_3 content was determined as reported previously [25]. Additions of respiration medium and mitochondria were made through a custom port installed in the top of the sphere with a polyethylene tube running down to the bottom of the chamber. After washing the chamber with buffer, preoxygenated buffer (100% O_2) was injected into the chamber, followed by the appropriate amount of mitochondrial stock solution. Subsequent additions were made through the injection port using a Hamilton syringe. The typical sequence of additions was inorganic phosphate (P_i, 3 mM), occasionally followed by a low concentration of ADP (50 μM) to generate the oxidized or O state. The low concentration of ADP was added during experiments in which P_i alone was insufficient to rapidly generate the O state. Glutamate and malate (5 mM each) was added to generate the substrate reduced or SR state. ADP (500 μM) was added to initiate the ATPs state. A small amount of sodium hydrosulfite was added to generate complete reduction and removal of oxygen or the CR state. Total volume of the reaction medium was limited to 2 ml, such that a noticeable mixing vortex was observed and the impinged white light beam was collimated in order to be completely contained within the sample. Oxygen consumption, NADH fluorescence, and mitochondrial membrane potential ($\Delta\Psi$) were measured in parallel experiments at 0.25 nmol of cytochrome aa_3/ml using previously established methods in a specially designed, water-jacketed reaction chamber [26]. Specifically, $\Delta\Psi$ was measured via tetraphenylphosphonium (TPP^+) distribution using a TPP^+ electrode (World Precision Instruments, KWIKTPP-2) in the respiratory suspension.