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Optical spectroscopy in turbid media utilizing an integrating sphere: mitochondrial chromophore analysis during metabolic transitions

David J. Chess^{a,*}, Eric Billings^a, Raúl Covian^a, Brian Glancy^a, Stephanie French^a, Joni Taylor^a, Heather de Bari^a, Elizabeth Murphy^b, and Robert S. Balaban^a

^aLaboratory of Cardiac Energetics, National Heart, Lung, and Blood Institute; National Institutes of Health; Bethesda, Maryland, 20892, USA

^bSystems Biology Center, National Heart, Lung, and Blood Institute; National Institutes of Health, Bethesda, Maryland, 20892, USA

Abstract

Recent evidence suggests that the activity of mitochondrial oxidative phosphorylation Complexes (MOPC) is modulated at multiple sites. Herein, a method of optically monitoring electron distribution within and between MOPC 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 to 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.

Keywords

mitochondria; spectroscopy; electron transport chain; ischemia-reperfusion

Over the last several years, there has been increased interest in measuring the enzymatic activity of mitochondrial oxidative phosphorylation Complexes (MOPC). This has been stimulated by the observation of numerous MOPC post-translational 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].

*To whom correspondence should be addressed: David J. Chess, National Heart, Lung, and Blood Institute, 10 Center Drive, Room B1D416, Bethesda, Maryland, 20892, USA, Phone: (301) 496-3658, Fax: (301) 402-2389, chessdj@mail.nih.gov.

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Methods to monitor MOPC activity include intact mitochondrial studies with inhibitors and artificial substrates, assays on extracted protein in solution [8], or 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 ($\Delta\Psi$), un-physiological 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 [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 MOPC [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. Though 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.

The purpose of the current study was to adapt the original methods of Chance, Halestrap, and the Wittenbergs to monitor 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 use of hydrogen peroxide, high maximum respiratory rates and respiratory control ratios, and rapid spectral acquisition rates (~100 msec). 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 chemically-induced 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 substrate reduction to ATP synthesis. This approach then was applied to

mitochondria isolated from perfused rabbit hearts following ischemia-reperfusion 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 Spectrafect® reflectance coating (model RTC-060-SF, LabSphere, Inc., North Sutton, NH, USA). A custom-made, cylindrical, water-jacketed, quartz cuvette (12 mm internal diameter, 20 mm outer diameter, 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, Inc.). 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. Also, conventional oxygen electrodes differentially absorbed light, thereby decreasing the bandwidth of our measurements. As such, in order 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 watts using a CrimeScope (model CS-16-500, Jobin-Yvon, Inc., Spex® Forensics Division, Edison, NJ, USA). After passing through a focusing assembly, incident light was delivered to the integrating sphere via a 1000- μ m diameter optic fiber (Ocean Optics, Inc., 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, Inc.). 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 measured the temperature of the mixing chamber with mitochondria (1 nmol cytochrome *aa₃*/mL) while the light was turned off and on for 5 minute 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 et al. [23] using high-power UV pulses on a similar preparation. A second 1000- μ m 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, Inc.). Spectra were collected at 100 ms integration using SpectraSuite software (Ocean Optics, Inc.) 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, 0.2 mM K₂EDTA, pH 7.1. The standard experimental buffer used for mitochondrial respiration contained (in mM): 125 KCl, 15 NaCl, 20 K-HEPES, 1 KEGTA, 1 K₂EDTA, 5 MgCl₂, pH 7.1 at 37° C. Sphere studies were conducted on mitochondrial

concentrations of 1 to 2 nmol cytochrome *aa₃*/mL which were found to remain in the linear domain of the system (see below). Cytochrome *aa₃* content was determined as previously reported [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, pre-oxygenated buffer (100% O₂) 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 ATP synthesis or 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 cytochrome *aa₃*/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.

Calibration of the integrating sphere

Optimization of the integrating sphere included determination of the frequency dependence of the pathlength, optimal protein concentration, and measurement of light leak. To determine the effective pathlength of the chamber, calibrated solutions of India ink with known absorbance versus frequency characteristics were added to the chamber with water and with 1 nmol cytochrome *aa₃*/mL suspension. The absorbance of the India ink was collected to determine the effective pathlength at each frequency in the suspension and the water solution (non-scattering case) as previously described by Rothstein et al. [26]. The pathlength dependence on frequency was nearly linear over this narrow bandwidth. For reference, this system had a 1.18 cm pathlength at 400 nm and 1.24 cm at 600 nm. The bandwidth of these experiments was limited by the light source spectrum. Using UV or IR enhanced light sources can expand the bandwidth. Although the inner surface of the sphere was highly reflective, the repeated reflections resulted in a new absorbance of the sphere alone on the order of 1 O.D., requiring a powerful input light source and sensitive detection system.

To determine optimal protein concentration, mitochondria were suspended in respiration buffer at linearly-increasing concentrations from 0.125 mg/mL to 8 mg/mL (which, in the heart, equates to the same concentration in nmol cytochrome *aa₃*/mL). The summation of absorbance values from 400-650 nm was determined for each protein concentration and normalized to 0.125 mg/mL. A linear increase in absorbance ratio was observed for concentrations at or below 2 mg/mL, above which there was noticeable deviation between the theoretical and experimental absorbance ratio. Though 2 mg/mL provided better signal-to-noise than 1 mg/mL, it also depleted the respiration medium of oxygen quickly, leading to anoxia-related changes in the cytochromes. Therefore, 1 mg/mL was selected for most, but not all, of the experiments.

Light leak, defined as incident light not passing through the sample (and therefore not contributing to sample absorbance), was likely caused by the curvature of the outer and inner walls of the cylindrical quartz cuvette. The magnitude of light leak was determined

using pure India ink. Dilution of the India ink revealed that with pure ink, the light collected was essentially independent of the absorbance of the sample and was taken to represent an estimate of the light leak around the chamber. It was assumed that the fraction of incident light composing the light leak was directly proportional to the intensity of the incident light. The light leak was modeled as a function of frequency and used to correct incident and experimental spectra as another “dark current.” This correction, like the dark current, only represented a few percent of the incident light, even in the 400 nm region.

Individual chromophore reduced minus oxidized spectra for linear regression fit

For spectral fitting, reduced minus oxidized difference spectra of the purified components were collected using a variety of techniques. However, all spectra were collected in the experimental chamber to assure fidelity between the reference and experimental data. Difference spectra rather than absolute oxidized and reduced spectra were used for this analysis since it eliminated any non-labile absorbance of the mitochondria from the analysis. In addition, the fully oxidized spectrum of some components was difficult to obtain.

The spectrum of flavin adenine nucleotide (FAD) was obtained from purified FAD and sarcosine dehydrogenase, which requires FAD as a cofactor. Some of the FAD absorbance may have represented other flavin mononucleotides. As such, this chromophore will be referred to as “*FN*”.

For the b_H species, the ubiquinone:cytochrome c oxidoreductase (Complex III) was purified from isolated porcine left ventricular mitochondria using anionic exchange chromatography. The purified enzyme (3-16 mg/mL) was stored in 50% (v/v) glycerol and 0.1% lauryl maltoside. Spectral isolation of the b_H chromophore was obtained from isolated complex in solution by adding ascorbate/potassium cyanide (to eliminate c_1 and contaminating cytochrome aa_3) and titrating sodium hydrosulfite to only reduce b_H .

The extraction of b_L was more difficult as many conditions altered the spectral properties of this chromophore. We reasoned that acquiring a spectrum of b_L as close as possible to the true mitochondrial conditions would be ideal. To accomplish this, we used the fact that the b_L redox state is highly dependent on the membrane potential. To isolate b_L using this property, we allowed a high concentration of mitochondria to run to anoxia to reduce all of the other chromophores. FCCP was then added to collapse the residual $\Delta\Psi$, resulting in a difference spectrum before and after FCCP with a maximum alpha peak at 565.5 nm, consistent with b_L .

Cytochrome c_1 and c spectra were collected using the method established by Estabrook [27].

Acquisition of cytochrome oxidase (Complex IV) spectra was complicated by the known existence of multiple species within the enzyme’s catalytic cycle. Specifically, the spectrally visible cytochrome a_3 -Cu_B intermediates are referred to by various nomenclature, but herein are denoted $a580$, $a607$, and $a605$, denoting the peak center positions. Isolated cytochrome oxidase derived from the anionic exchange chromatography was titrated with hydrogen peroxide, generating the $a580$ species as described by von der Hocht et al. [28]. The $a605$ and $a607$ species were observed during chemical reduction and ATP synthesis, respectively. The $a605$ species was produced by treating isolated cytochrome oxidase with sodium hydrosulfite. The $a607$ species derived from hydrogen peroxide titration was contaminated by the $a580$ species. As such, the $a607$ reference spectrum was produced by red-shifting the $a605$ spectrum 2 nm. The line shape of these species agreed well with both the purified cytochrome oxidase as well as the intact mitochondrial spectra, as seen in the residuals of the fits.

The fitting matrix used in these studies is provided in the Supplemental Data.

Raw data analysis and spectral fitting

Within a given mitochondrial respiratory time course experiment, an average of 2,500-3,500 spectra containing raw light intensity values were collected by the rapid-scanning spectrometer at a rate of 1 spectrum/100 msec. The 100 msec integration time was selected since this was on the order of the mixing time in the chamber. Using custom software written in the IDL programming language (IDL Version 6.3), raw light intensity (across the entire spectral range of the detector, 348.89-745.99 nm) first was corrected by subtraction of both the dark current of the spectrometer and the light leak. Absorbance then was calculated using the following equation:

$$A = -\log_{10}(E_{\lambda}/R_{\lambda}),$$

where E_{λ} represents the raw light intensity of the experimental spectrum and R_{λ} the incident light intensity at a particular wavelength. With the absorbance spectra compiled into a single matrix, a difference between any two spectra could be obtained by selecting points within the time domain. Signal-to-noise of these differences was improved by averaging between 10-500 spectra. Fully oxidized spectra were obtained by adding Pi, and in some cases a small amount of ADP, to mitochondria in the absence of carbon substrates. Fully reduced spectra were obtained using a small addition of sodium hydrosulfite to the chamber. Difference spectra then were fitted with a linear least squares regression using the isolated reference spectra, which were zeroed at the 630 nm position (a known isosbestic point for the mitochondrial chromophores). This was accomplished by assuming the observed difference spectrum was the simple sum of all of the reference difference spectra using the linear relationship: mitochondrial difference spectrum = $FN + b_H + b_L + c_1 + c + a580 + a607$ (or $a605$ in the case of chemical reduction). Baseline correction was performed by adding a linear term with a variable fixed slope and offset; however, this term provided only a minor improvement to the overall fit. Residuals of this fit were generally much less than 5% of the primary data depending on the signal-to-noise of the experiment. Initially, the spectral fit was applied over the entire spectrometer bandwidth (348.89-745.99 nm). However, the limited spectral dispersion in the Soret band compromised the fitting routine during small changes in absorbance. Subsequent statistical analysis [29] revealed poor selectivity in this region while the beta/alpha region was superior in resolving the chromophores of interest. Thus, the Soret band was used only as qualitative confirmation of redox directional changes observed in the visible region.

Principal Component Analysis (PCA)

Principal Component Analysis (PCA) was applied as an unbiased method to identify the dominant sources of variation in the spectra over time and to confirm that the reference spectra were sufficient to represent the response of the MOPC chromophores. PCA was used for its ability to decompose the conditioned time series data into orthogonal principal components that individually account for a known amount of the variation in the data [30,31]. As mathematical constructs, the principal components represent the correlated responses of several chromophores to the addition of reagents. Multiple linear regression (MLR) was performed for each (orthogonal) principal component using the reference spectra and the residual error was examined. This provides a measure of the minimum variation, which may not be explained using the same fitting matrix as used in the normal analysis. The residuals from this analysis are therefore outside the scope of the original linear model.

The top three principal components explain roughly 98.9% of the variation observed while additional components explain no more than 0.016% individually. Comparison of the principal components and reference spectra indicates that no principal component is dominated by a single MOPC component. Isolated features of several reference spectra are evident in one or more principal components. For example, the 550 nm peak of cytochrome *c* and the combined 605 and 607 nm peaks of cytochrome oxidase are evident in the first four principal components.

Isolated mitochondrial studies from rabbit hearts subjected to ischemia-reperfusion

To ensure that temporal differences in tissue processing were not factors in altering mitochondrial function, two rabbit hearts were perfused simultaneously in a dual-cannulation Langendorff perfusion apparatus with identical perfusion pressures. Two hearts from New Zealand white rabbits were harvested and cannulated simultaneously. After a 30-minute baseline perfusion period, one heart was subjected to 45 minutes of global, no-flow ischemia, while the other was continued under control conditions. During the ischemic period, the heart was placed in a water-jacketed chamber filled with perfusion buffer maintained at 38° C. Upon completion of ischemia, the heart was removed from the chamber and normal perfusate flow was restored. Reperfusion was performed for 30 min., after which both hearts were rapidly perfused with ice-cold mitochondrial isolation buffer and removed from the perfusion apparatus. Following removal of the right ventricle and visible fat and connective tissue, the left ventricles were weighed. A 20% homogenate was prepared by mincing the left ventricle in isolation buffer followed by Vertishearing (10 sec. at 160 rpm). Mitochondria were isolated using a trypsin digestion and differential centrifugation as described previously [24] and subjected to paired respiration/NADH fluorescence/ and integrating sphere experiments. No attempt to collect a non-trypsin (or subsarcolemmal) population was attempted.

Calculation of redox potentials, free energies, and conductance

Absorbance data collected from the integrating sphere and subsequent spectral fitting provided the redox state (in percent reduced) of each chromophore throughout a respiration experiment. Given the percent reduction, the redox potential of the chromophores could be determined using the following equation:

$$E = E^{\circ} + 2.3 (RT/nF) \log_{10} ([ox] / [red])$$

where E° is the midpoint potential, R is the gas constant (1.987×10^{-3} kcal/mol K), T is the temperature in Kelvin, n is the number of electrons transferred between the two species of the redox pair (Oxidized + $ne^{-} \leftrightarrow$ Reduced), and F is the Faraday constant (23.062 kcal/V). The midpoint potentials used for the mitochondrial redox pairs can be found below [32-35]:

Redox Pair	Midpoint Pot. (mV)
NAD ⁺ /NADH	-320
Cytochrome <i>c</i> ₁	+230
Cytochrome <i>c</i>	+270
Cytochrome <i>a</i> 605	+280
O ₂ /H ₂ O	+820

From the redox potentials, the free energy associated with a two-electron transfer between mitochondrial redox components could be determined:

$$\Delta G = -nF(\Delta E_h + z\Delta\Psi) - mF\Delta\Psi$$

where n is the number of electrons transferred, F is the Faraday constant, ΔE_h is the redox potential difference (in mV), $\Delta\Psi$ is the mitochondrial membrane potential (in mV), z is the distance traveled by electrons across the inner mitochondrial membrane relative to the matrix side, and m is the number of protons pumped (if applicable). The electron transfer steps examined and their corresponding stoichiometries were as follows:

NAD ⁺ /NADH to Cyt <i>c</i> :	$n = 2, z = 1, m = 8$
Cyt <i>c</i> to Cyt <i>a</i> :	$n = 2, z = 0, m = 0$
Cyt <i>c</i> to O ₂ /H ₂ O:	$n = 2, z = -1, m = 2$
Cyt <i>a</i> to O ₂ /H ₂ O:	$n = 2, z = -1, m = 2$
NAD ⁺ /NADH to O ₂ /H ₂ O:	$n = 2, z = 0, m = 10$

Conductance was then calculated using the measured oxygen consumption (current) and the free energies associated with electron transfer across different segments of the electron transport chain:

$$\text{Conductance} = (\text{O}_2 \text{ consumption}) / \Delta G$$

This is analogous to determining the conductance of an electrical circuit, derived from Ohm's law ($V = IR$).

Statistical Analysis

For experiments performed using isolated porcine heart mitochondria, differences in chromophore redox state between the substrate reduced (SR) and ATP synthesis (ATPs) states were assessed using a paired Student's *t*-test. For studies involving mitochondria isolated from rabbit hearts subjected to control conditions or ischemia-reperfusion, differences in chromophore redox state and electron transport chain conductance were assessed using an unpaired Student's *t*-test. Results were considered significant at a $p < 0.05$.

Results

Light collection using an integrating sphere

Light scattering is an inherent property of turbid suspensions such as isolated mitochondria. Using conventional spectroscopic techniques, it is difficult to make accurate absorbance measurements when scattering dominates the trajectory of photons. An integrating sphere, with a center-mounted sample chamber as illustrated in Fig. 1A-B, can mitigate the effects of scattering by effectively sampling the scattered light from the sample [36,37]. These scattering effects in a conventional split-beam spectrograph are presented in Fig. 2A-D. The high apparent absorbance of the mitochondrial suspension (~2 O.D.) is dominated by scattering, as witnessed by the nearly linear increase in absorbance with decreasing wavelength over this rather narrow bandwidth (Fig. 2A). An oxidized versus chemically reduced difference spectrum (Fig. 2B) has low signal-to-noise for the mitochondrial chromophores due to the dominance of scattered light, especially in the ~400 nm Soret

region. When solubilized in phosphate-buffered 2% Triton-X solution, the same mitochondrial suspension exhibited reduced scattering [25], lower overall absorbance (~ 0.25 O.D.), and greatly improved signal-to-noise of the mitochondrial chromophores (Fig 2C-D). These data demonstrate that scattering, not the chromophore extinction coefficients, limits detection. The center-mounted integrating sphere shown in Fig. 1A, where the scattered light is sampled and not lost, improves the signal-to-noise and spectral dynamic range of the measurements. Furthermore, the integrating sphere system permitted simultaneous monitoring of the Soret, beta, and alpha absorbance bands (~ 400 -630 nm) of the mitochondrial chromophores with little interference from scattering effects. Consequently, absolute and difference spectra of intact mitochondria reveal spectral quality approaching that of a detergent-treated sample in a conventional spectrometer (Fig. 2E-F).

The use of a center-mounted sample chamber in an integrating sphere introduces several issues that need to be corrected for in quantitative studies. The necessary corrections are for the frequency dependence of optical pathlength, the light scattered before entering the sample (especially with the water-jacketed system used in this study), and the spectral characteristics of the integrating sphere reflective surface [26,36,37]. These are evaluated and discussed in the Materials and methods section.

Spectral fitting

Individual chromophore spectra were obtained by deconvolution of the mitochondrial difference spectrum using multiple linear least squares [38,39]. It is important to note that this approach permits the quantitative deconvolution of complex spectra, providing the accuracy of the reference spectra used [29]. The reduced minus oxidized difference spectra used were as follows: for Complex III, cytochrome b_{low} (b_L), cytochrome b_{high} (b_H), and cytochrome c_1 (c_1); for Complex IV, ferryl cytochrome a_3 ($a580$), peroxy cytochrome a_3 ($a607$) – as initially described by Wikstrom [40,41] – and the combined cytochrome aa_3 ($a605$). Spectra from flavins (FN) and cytochrome c (c) were also included. These were obtained primarily from purification of mitochondrial extracts, specialized conditions in intact mitochondria, and commercial sources. All spectra were collected within the integrating sphere system to assure that the collection conditions were identical to those used with intact mitochondria. Reference chromophore spectra were normalized at the 630 nm isosbestic point to correct for baseline differences (Supplemental Table 1). Where possible, the fit coefficients for each spectral element were normalized to the fully oxidized (0) and fully reduced state (100). Due to the significant chromophore spectral overlap in the Soret band, its utility for fitting was limited based on standard selectivity measures [29]. Thus, we restricted the quantitative analysis to the region from approximately 465 to 630 nm and used the Soret band for qualitative confirmation of the results. Principal Component Analysis (PCA) was applied as an unbiased method to identify the dominant features in the spectral data and to validate that the linear regression using the reference spectra could accurately reproduce the MOPC redox changes. The top three principal components explained roughly 98.9% of the variation observed while additional components accounted for no more than 0.016% individually (Supplemental Fig. 4). The contribution of each component varied in response to the various perturbations during a typical respiration experiment (Supplemental Fig. 5), and each component could be fit with a low residual error using the chromophore reference spectra (Supplemental Fig. 6).

MOPC redox transitions

Our initial studies evaluated four functional states of the mitochondrial suspensions. The conditions were generally collected in a single run to facilitate analysis of the difference spectra. The conditions were fully oxidized (O), substrate reduced (SR), ATP synthesis (ATPs), and fully reduced with sodium hydrosulfite (CR). These conditions are outlined in

Fig. 3A with the relative oxygen tension of the chamber. A complete data set running from O to CR states is provided in video format with the raw data (Supplemental Movie #1), fit data (Supplemental Movie #2), and the residuals (Supplemental Movie #3). These data were time averaged to a 1-second time resolution to improve presentation.

The first difference spectrum presented is the SR minus O state with the addition of 5 mM glutamate and malate to mitochondria (Fig. 3B, C). The linear least squares analysis revealed a strong reduction in FN , b_H , b_L , c_1 , c , and an increase in the concentrations of $a580$ and $a607$. The residuals for this experiment and for over 20 spectral runs were $< 5\%$ of the integrated intensity of the region of interest. The detection of the $a580$ and $a607$ species within Complex IV was unexpected and not reported in previous studies, to our knowledge, in intact mitochondria during these classical state transitions. It is important to note that the $a607$ component resulted in a significant improvement in the fit compared to the fit using the $a605$ component in this transition. A full bandwidth SR-O spectrum, along with other transitions, is presented in Supplemental Fig. 1.

The next stage is the conversion from SR to ATPs (Fig. 3D, E). A difference spectrum between these two respiratory states demonstrated a reduction in cytochromes b_H , c_1 , and c , and an oxidation of FN and b_L . A redox shift in which one chromophore goes more reduced while an adjacent chromophore becomes more oxidized is termed a redox “crossover” [17]. A redox crossover was consistently found within Complex III, where b_L became more oxidized while c_1 became more reduced, in agreement with previous reports [19,42]. In a prior study on P_i alone [42], it was suggested that the crossover between b_L and cytochrome c was specific for a P_i addition to initiate ATP production. As shown in this study, either ADP- or P_i -induced ATP production causes a crossover between b_L and cytochrome c in this preparation, likely driven by the decrease in redox state of b_L in response to the drop in.

An interesting transition was observed within Complex IV, in which the $a580$ concentration decreased while that of $a607$ increased upon initiation of ATPs. This is somewhat surprising since the overall reduction level of Complex IV was believed to increase with ATPs [10,11]. However, these results are consistent with a decrease in the concentration of $a580$, the a_3 - Cu_B intermediate which accepts and transfers the final electron for the reduction of oxygen to water [43,44].

Similar observations were made in over 20 independent experiments in pig heart mitochondria. The mean position of the raw Complex IV peak absorbance was 606.5 ± 0.4 for these studies, consistent with the fitting analysis requiring a 607 nm absorbance. Analogous results were obtained in rabbit heart (Supplemental Fig. 2) and pig skeletal muscle mitochondria (Glancy et al., *Biochemistry*, in press).

The final transition evaluated in this study was the full chemical reduction of the mitochondria (Fig. 3F, G). As in the difference spectrum between SR and O, a large reduction in FN , b_H , c , and c_1 was observed. In addition, an increase in $a605$ concentration was found with CR. The increase in the reduction of b_L was modest, while inclusion of the $a580$ and $a607$ species was not required for adequate fitting. The mean absorbance in the raw data in the Complex IV region was 605.2 ± 0.2 nm ($n = 12$) for CR, consistent with the dominance of the 605 nm Complex IV species under oxygen depleted conditions [44-46]. We found the CR method to provide the most reproducible reduction, especially of Complex IV, and thus was used in our quantitative studies to determine the 100% reduction level for some species. The better reproducibility of CR relative to simply allowing mitochondria to reach anoxia was likely due to the fact that this version of the chamber is not sealed and absolute anoxia is difficult to achieve.

A summary of the mitochondrial chromophore reduction levels in the SR and ATPs states for pig heart mitochondria is shown in Table 1. FN and b_L were assumed to be 100% reduced in the SR state, where λ_{580} also was assumed to be at its maximum due to the high membrane potential and excess of glutamate-malate used as substrate. Similar SR-O, ATPs-SR, and CR-O spectral responses were observed in rabbit heart mitochondria (Supplemental Fig. 2) and in pig skeletal muscle mitochondria (Glancy, Chess, and Balaban, unpublished observation). The full-bandwidth difference spectra for the transitions are presented in Supplemental Fig. 1.

Effect of intermediate ATP synthetic rates on MOPC redox states

To examine the intermediate rates of respiration on cytochrome redox state and membrane potential, we determined oxygen consumption rate (J_o), mitochondrial membrane potential ($\Delta\Psi$), and the redox state of mitochondrial chromophores in paired experiments in which the extra-mitochondrial ATP hydrolysis rate was systematically incremented by addition of an exogenous ATPase (apyrase) [47]. For each paired experiment, P_i and glutamate/malate were added, followed by 5 mM ATP. Upon subsequent addition of graded amounts of apyrase, J_o and ATP synthetic rate increased [47]. The difference spectra of the various concentrations of apyrase minus SR are presented in Fig. 4A. The relationship between $\Delta\Psi$ and J_o ($J_o = 7 \cdot \Delta\Psi + 1389$; units are $J_o = \text{nmol O}_2/\text{min}/\text{nmol cytochrome } aa_3$; $\Delta\Psi = \text{mV}$) (inset, Fig. 4A) was linear, consistent with previous studies. Force-flow analysis of Complex IV (Fig. 4B-F) indicated a linear relationship between the concentration of λ_{607} and J_o or $\Delta\Psi$, with oxygen consumption increasing in proportion to the λ_{607} absorbance. Thus, the level of λ_{607} can be used as an indicator of flow, assuming a fixed conductance in Complex IV under a particular condition. A similar relationship between λ_{607} concentration and uncoupled respiration using varying doses of FCCP was also obtained (Fig. 4D) implying that oxidative phosphorylation was not required to maintain this relationship. Surprisingly, the relationship between $[\lambda_{580}]$ and J_o (Fig. 4E) and $\Delta\Psi$ (Fig. 4F) was non-linear, with $[\lambda_{580}]$ dropping precipitously during the initiation of respiration ($\sim 100 \text{ nmol O}_2/\text{min}/\text{nmol cytochrome } aa_3$ and $\sim -180 \text{ mV } \Delta\Psi$) and then remaining essentially constant up to V_{max} .

Effect of ischemia-reperfusion on mitochondrial driving forces and conductances

We undertook a study to evaluate the systemic effects of the extensively-studied cardiac ischemia-reperfusion perturbation on the driving forces and reaction conductance network of oxidative phosphorylation in the rabbit heart. Using the redox states of the chromophores (as estimates of MOPC redox state), $\Delta\Psi$ and J_o , the overall driving force and current through numerous steps within oxidative phosphorylation could be determined.

We selected the rabbit heart ischemia-reperfusion (I/R) model as a perturbation to mitochondrial function [48,49] since it has been extensively studied and the rabbit heart is large enough to generate an adequate sample for optical analysis. The fitting matrix of chromophores derived for the pig heart mitochondria worked equally well for the rabbit heart mitochondria (Supplemental Fig. 2) and the behavior of the chromophores was similar to that established in the pig heart. In these experiments, two simultaneously-isolated, Langendorff-perfused rabbit hearts were subjected to normal perfusion or prolonged global no-flow ischemia (45 min.) followed by reperfusion (30 min.). These conditions were selected from literature and preliminary studies to generate a modest but significant impact on cardiac and isolated mitochondrial function [50,51]. Upon completion of the perfusion, mitochondria were isolated from the left ventricles of both hearts (with EGTA to ensure no Ca^{2+} uptake) and subjected to spectroscopic analysis in the integrating sphere. Mitochondrial $\Delta\Psi$, J_o , and NADH fluorescence were determined in paired experiments as previously described. Cytochrome aa_3 content was used for normalization as previously described [25]. Importantly, no loss of cytochrome c was observed in this study comparing

the fully reduced spectra of the control and I/R treated mitochondria using the linear fitting routines. An overlay of the I/R and control fully reduced spectra is presented in Supplemental Fig. 7.

The impact of the I/R treatment on overall mitochondrial function was significant ($p < 0.05$) and consistent with the literature, with the ATPs J_o decreasing from 256.00 ± 7.83 nmol O_2 /min/nmol cytochrome aa_3 in control to 169.50 ± 16.87 after I/R. In addition, the SR J_o was 38.0 ± 3.0 nmol O_2 /min/nmol cytochrome aa_3 in control and significantly higher at 52.0 ± 4.0 after I/R, suggesting an increase in proton leak after I/R. Consistent with this notion was a significant depolarization in SR $\Delta\Psi$ from -179 ± 1 mV in control to -162 ± 2 mV in I/R. The NADH reduction level during SR was $82 \pm 3\%$ in control and $81 \pm 2\%$ in I/R while in ATPs the control level was $31 \pm 1\%$ and in I/R was $45 \pm 4\%$.

The optical absorption protocol was similar to the pig heart mitochondrial studies using an initial oxidation period (no carbon substrate and P_i) followed by SR (5 mM glutamate/malate), ATPs (1.3 mM ADP), and CR. A summary of the optical absorption results for the rabbit heart I/R experiments is presented in Supplemental Table 2. The SR-O redox levels for NADH and b_H were similar in both conditions; however, c_1 , c , and $a607$ were more reduced (consistent with the proton leak and increased SR respiratory rate).

During ATPs, J_o was suppressed in I/R mitochondria; however, $\Delta\Psi$ was essentially identical (147 ± 1 mV in control and 145 ± 2 mV after I/R). The control and I/R mitochondria exhibited redox crossovers in Complex III and Complex IV during ATPs; however, the magnitude of the redox transitions was lower in I/R mitochondria. Taking Complex IV alone, it was surprising that despite a similar $[a607]$ and $\Delta\Psi$ that the respiratory rate was suppressed in I/R, suggesting an inhibition of Complex IV activity.

To examine the effect of I/R on the entire oxidative phosphorylation network, we used the redox state of NADH, FN , and cytochromes in conjunction with $\Delta\Psi$ and the respiratory rate to calculate the effective conductance of different regions of the oxidative phosphorylation network using the Onsager approach [52,53], where the apparent conductance of the reaction is proportional to the force-flow relationship. In the case of oxidative phosphorylation elements, conductance can be calculated by dividing J_o by the net driving force for the reaction ($\Delta\Psi$, redox states, and relevant ΔG_0). Using this approach, we hoped to identify specific regions of the reaction network that were inhibited by the I/R challenge. These calculations are presented in Table 2 for the ATPs state (or maximum respiration) conditions for 5 regions of the network with the equations used to calculate their relative conductances. These 5 regions are as follows: the reaction from NADH through cytochrome c , from cytochrome c to Complex IV, from cytochrome c to oxygen, from Complex IV to oxygen, and for the entire oxidative phosphorylation reaction from NADH to oxygen. The free energies for some redox species (ΔG_0) are either poorly defined or full-scale oxidation and reduction values are not available (e.g., $a580$, $a607$). In these cases, it was assumed that the available free energy was proportional to the concentration of these elements as reflected in the absolute absorbance. As seen in Table 2, the conductances of all of the regions examined were inhibited to nearly the same extent as respiration by I/R treatment. These data suggest a novel hypothesis that the inhibition of oxidative phosphorylation by I/R is distributed across the entire network and is not focused at any one oxidative phosphorylation Complex.

Discussion

Using a center-mounted sample in an integrating sphere and a rapid-scanning optical spectroscopy system, high-quality dynamic optical spectra can be collected from respiring

intact mitochondria. A matrix of mitochondrial chromophore reference spectra has been developed that adequately fits the entire beta and alpha bands of the redox-sensitive chromophores. Using this approach, the $\lambda 580$ and $\lambda 607$ redox species within Complex IV were found to be dynamically modulated under “physiological” perturbations in several mitochondrial preparations. Previously, these were only observed in intact mitochondria under extreme conditions with ferricyanide for oxidation or ascorbate plus tetramethylphenylenediamine (TMPD) [41,43]. The transitions between the $\lambda 607$ to $\lambda 580$ as well as the $\lambda 580$ to fully oxidized (O) state are dependent on $\Delta\Psi$ [43,54] (Supplemental Fig. 3) and phosphorylation potential [55]. We hypothesize that during the ATPs transition, $\Delta\Psi$ depolarization favors the addition of the 4th electron to $\lambda 580$ more than the 3rd electron to $\lambda 607$. Thus, to match $\lambda 580$ conversion to O, the [$\lambda 607$] must increase. The linear relationship between $\lambda 607$ versus $\Delta\Psi$ as well as J_o , while $\lambda 580$ reaches a minimum during the initial increase in J_o and depolarization (Fig. 4B-F), leads to the speculation that the addition of the 3rd electron to $\lambda 607$ is the rate-limiting step within Complex IV under these conditions. That is, once the $\lambda 580$ is formed by the reduction of $\lambda 607$ during ATP production (and a low) it immediately acquires the 4th electron and reduces oxygen, resulting in the near constant and low [$\lambda 580$]. Addition of ATP to the SR condition decreased $\lambda 580$ absorbance; however, this effect is abolished upon administration of oligomycin (data not shown), suggesting that the effect of ATP is due to the action of Complex V, which results in a small depolarization under these conditions due to contaminating ATPase activity. Similar results were obtained by Wikstrom [55] in evaluating the effect of the G_{ATP} and the occupancy of these two states. The original observation of a net reduction of Complex IV during the transition from SR to ATPs was likely dominated by the reduction of $\lambda 607$ while the $\lambda 580$ was not observed [10,11].

It has been well known that I/R inhibits mitochondrial oxidative phosphorylation; however, the precise mechanisms are debated [50,51,56-58]. We used this technology to probe many of the reactions within oxidative phosphorylation to establish which reaction might be specifically altered by I/R challenge. Surprisingly, we found that despite a 34% reduction in the maximum rate of ATP production with I/R, the calculated conductances for all of the steps evaluated were inhibited to essentially the same extent. This was most notable within Complex IV during ATPs, where the [$\lambda 607$] and $\Delta\Psi$ were essentially the same in control and I/R conditions while [$\lambda 580$] was much higher in I/R (Supplemental Table 2). Thus, despite similar or increasing driving forces (redox state and $\Delta\Psi$), there was a decrease in J_o , signifying decreased efficiency of electron transfer within Complex IV. We speculate that I/R decreased the kinetics of acceptance of electrons by $\lambda 580$ within Complex IV, slowing the respiratory rate along with other steps within the metabolic cascade of oxidative phosphorylation. It should be noted that we did not attempt to evaluate a trypsin and non-trypsin isolated mitochondrial pool in these studies and the results could be different from a non-trypsin or so-called subsarcolemmal population of mitochondria that may be even more susceptible to IR effects [59-65].

As discussed above, a limitation of this approach is the need to isolate mitochondria from the heart, which can alter their activities [66] as well as select only subsets of mitochondrial pools. Though we demonstrated superb experimental control of isolated mitochondria in the integrating sphere, we speculated whether the optical approaches and reference spectra could be applied to more physiological models, such as the saline perfused heart. In preliminary studies, we used the isolated perfused rabbit heart described in previous studies [42,67] in the Langendorff mode to evaluate if the optical elements determined in the isolated mitochondria would apply to the intact heart. We used the same light source and detector as in the isolated mitochondria studies but simply impinged light across the heart wall using a fiber optic and collected the transmitted light across the wall with another fiber optic as previously described [67]. We hypothesized that the only difference between the

isolated mitochondria and the intact saline perfused heart would be the myoglobin absorbance at low oxygen tensions. An oxygenated-deoxygenated myoglobin difference spectrum was obtained using commercially available myoglobin (Sigma, horse heart myoglobin, M-1882). After pre-reducing the myoglobin with a small amount of sodium hydrosulfite, oxygenated and deoxygenated spectra were then collected. In previous studies [67], the pre-reduction of the commercial source was not collected resulting in a poor representation of the oxygenated/deoxygenated myoglobin spectrum. The myoglobin spectrum was added to the fitting matrix for the heart analysis. Using this approach, we collected optical spectra from 4 different intact perfused hearts. In these studies, spectra were collected from control or cyanide-treated hearts to evaluate the reduced mitochondria alone [42], while spectra from control versus ischemic hearts were evaluated to observe both the mitochondrial reduction as well as myoglobin deoxygenation. Representative fits of these spectra are presented in Supplemental Fig. 8. The fits of the cyanide spectrum (Supplemental Fig. 8A-B), using a605 for cytochrome oxidase, resulted in similar small residuals as found in the isolated mitochondria. Importantly, under these conditions, essentially no myoglobin was needed (although it was included in the fitting process). In contrast, with ischemia (Supplemental Fig. 8C-D), a large myoglobin absorbance was detected in addition to the mitochondrial chromophores; however, the residuals of the fit again remained small. These data suggest that the spectral elements determined in the isolated mitochondria work well in the intact heart and that, with the addition of myoglobin spectra, the mitochondrial cytochrome redox states can be analyzed in the intact heart using this approach.

In conclusion, the data presented herein suggest that mitochondria respond to increased energy demand via steady-state changes in reducing equivalent distribution, potentially through alterations in post-translational modifications (PTMs), super-Complex structure, or even the structure of the mitochondrion itself. Hopefully, use of this optical approach of monitoring Complex activity in intact mitochondria in combination with the evaluation of PTMs and molecular structures may shed more light on the overall regulation of oxidative phosphorylation under both control and different pathophysiological states. It is also important to note that in theory these non-destructive approaches could be applied to mitochondria-rich tissues *in vivo*, providing an estimate of mitochondrial Complex activity under true physiological conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

D.J.C., R.C., B.G., E.M., H. de B., and R.S.B. designed experiments. R.C. and B.G. aided in conceptual development and data analysis. E.B. completed principal component analysis on the raw spectral data. R.S.B. composed the custom-written IDL software. D.J.C. carried out integrating sphere respiration experiments, spectral fitting, and data analysis. S.F. isolated the porcine heart mitochondria and performed respiration experiments. J.T. assisted with the perfused rabbit heart experiments. D.J.C., R.C., and S.F. isolated rabbit heart mitochondria. D.J.C. and R.S.B. wrote the manuscript. All authors contributed to manuscript revision. There are no competing financial interests to disclose.

References

- [1]. Stucki JW, Lehman LH, Siegel E. Acylation of proteins by myristic acid in isolated mitochondria. *J. Biol. Chem.* 1989; 264:6376–6380. [PubMed: 2703494]
- [2]. Covian R, Balaban RS. Cardiac mitochondrial matrix and respiratory complex protein phosphorylation. *Am. J. Physiol Heart Circ. Physiol.* 2012; 303:H940–H966. [PubMed: 22886415]

- [3]. Hurd TR, Costa NJ, Dahm CC, Beer SM, Brown SE, Filipovska A, Murphy MP. Glutathionylation of mitochondrial proteins. *Antioxid. Redox. Signal.* 2005; 7:999–1010. [PubMed: 15998254]
- [4]. Murphy E, Kohr M, Sun J, Nguyen T, Steenbergen C. S-nitrosylation: a radical way to protect the heart. *J. Mol. Cell Cardiol.* 2012; 52:568–577. [PubMed: 21907718]
- [5]. Castro L, Demicheli V, Tortora V, Radi R. Mitochondrial protein tyrosine nitration. *Free Radic. Res.* 2011; 45:37–52. [PubMed: 20942571]
- [6]. Schapira AH. Mitochondrial diseases. *Lancet.* 2012; 379:1825–1834. [PubMed: 22482939]
- [7]. Balaban RS. Modeling mitochondrial function. *Am. J. Physiol Cell Physiol.* 2006; 291:C1107–C1113. [PubMed: 16971500]
- [8]. Birch-Machin MA, Turnbull DM. Assaying mitochondrial respiratory complex activity in mitochondria isolated from human cells and tissues. *Methods Cell Biol.* 2001; 65:97–117. [PubMed: 11381612]
- [9]. Schagger H, von Jagow G. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.* 1991; 199:223–231. [PubMed: 1812789]
- [10]. Chance B, Williams GR. Respiratory enzymes in oxidative phosphorylation. III. The steady state. *J. Biol. Chem.* 1955; 217:409–427. [PubMed: 13271404]
- [11]. Chance B, Williams GR. Respiratory enzymes in oxidative phosphorylation. II. Difference spectra. *J. Biol. Chem.* 1955; 217:395–407. [PubMed: 13271403]
- [12]. Chance B, Williams GR. The respiratory chain and oxidative phosphorylation. *Adv. Enzymol. Relat Subj. Biochem.* 1956; 17:65–134. [PubMed: 13313307]
- [13]. Chance B, Williams GR. Respiratory enzymes in oxidative phosphorylation. IV. The respiratory chain. *J. Biol. Chem.* 1955; 217:429–438. [PubMed: 13271405]
- [14]. Halestrap AP. The nature of the stimulation of the respiratory chain of rat liver mitochondria by glucagon pretreatment of animals. *Biochem. J.* 1982; 204:37–47. [PubMed: 7115329]
- [15]. Halestrap AP. Stimulation of the respiratory chain of rat liver mitochondria between cytochrome c1 and cytochrome c by glucagon treatment of rats. *Biochem. J.* 1978; 172:399–405. [PubMed: 210759]
- [16]. Beauvoit B, Chance B. Time-resolved spectroscopy of mitochondria, cells and tissues under normal and pathological conditions. *Mol. Cell Biochem.* 1998; 184:445–455. [PubMed: 9746339]
- [17]. Chance B, Hess B. Metabolic control mechanisms. II. Crossover phenomena in mitochondria of ascites tumor cells. *J. Biol. Chem.* 1959; 234:2413–2415. [PubMed: 13809103]
- [18]. Hollis VS, Palacios-Callender M, Springett RJ, Delpy DT, Moncada S. Monitoring cytochrome redox changes in the mitochondria of intact cells using multi-wavelength visible light spectroscopy. *Biochim. Biophys. Acta.* 2003; 1607:191–202. [PubMed: 14670609]
- [19]. Kim N, Ripple MO, Springett R. Measurement of the mitochondrial membrane potential and pH gradient from the redox poise of the hemes of the bc1 complex. *Biophys. J.* 2012; 102:1194–1203. [PubMed: 22404942]
- [20]. Wittenberg BA, Wittenberg JB. Oxygen pressure gradients in isolated cardiac myocytes. *J. Biol. Chem.* 1985; 260:6548–6554. [PubMed: 3997835]
- [21]. Wittenberg BA, Wittenberg JB. Myoglobin-mediated oxygen delivery to mitochondria of isolated cardiac myocytes. *Proc. Natl. Acad. Sci. USA.* 1987; 84:7503–7507. [PubMed: 3118370]
- [22]. Brown GC, Brand MD. Thermodynamic control of electron flux through mitochondrial cytochrome bc1 complex. *Biochem. J.* 1985; 225:399–405. [PubMed: 2983670]
- [23]. Joubert F, Fales HM, Wen H, Combs CA, Balaban RS. NADH enzyme-dependent fluorescent recovery after photobleaching (ED-FRAP): applications to enzyme and mitochondrial reaction kinetics, in vitro. *Biophys. J.* 2004; 86:629–645. [PubMed: 14695307]
- [24]. Territo PR, Mootha VK, French SA, Balaban RS. Ca(2+) activation of heart mitochondrial oxidative phosphorylation: role of the F(0)/F(1)-ATPase. *Am. J. Physiol Cell Physiol.* 2000; 278:C423–C435. [PubMed: 10666039]

- [25]. Balaban RS, Mootha VK, Arai A. Spectroscopic determination of cytochrome c oxidase content in tissues containing myoglobin or hemoglobin. *Anal. Biochem.* 1996; 237:274–278. [PubMed: 8660576]
- [26]. Rothstein EC, Carroll S, Combs CA, Jobsis PD, Balaban RS. Skeletal muscle NAD(P)H two-photon fluorescence microscopy in vivo: topology and optical inner filters. *Biophys. J.* 2005; 88:2165–2176. [PubMed: 15596503]
- [27]. Estabrook RW. The spectral and enzymatic characterization of cytochrome c1 of liver mitochondria. *J. Biol. Chem.* 1958; 230:735–750. [PubMed: 13525391]
- [28]. von der Hocht I, van Wonderen JH, Hilbers F, Angerer H, MacMillan F, Michel H. Interconversions of P and F intermediates of cytochrome c oxidase from *Paracoccus denitrificans*. *Proc. Natl. Acad. Sci. U. S. A.* 2011; 108:3964–3969. [PubMed: 21368144]
- [29]. Faber NM, Ferre J, Boque R, Kalivas JH. Quantifying selectivity in spectrophotometric multicomponent analysis. *Trends Anal. Chem.* 2003; 22:352–361.
- [30]. Smits RP, Jansonius NM, Stavenga DG. Dependence of receptor potential and redox state of mitochondria cytochromes on oxygen fraction measured in the blowfly eye in vivo. *J. Comp. Physiol.* 1995; 177:105–110.
- [31]. Zupan G. A method for dynamic spectrophotometric measurements in vivo using principal component analysis-based spectral deconvolution. *Pflügers Arch. -Eur J. Physiol.* 2003; 447:109–119. [PubMed: 12920600]
- [32]. Wikström M, Verkhovsky MI. Mechanism and energetics of proton translocation by the respiratory heme-copper oxidases. *Biochim. Biophys. Acta.* 2007; 1767:1200–1214. [PubMed: 17689487]
- [33]. Kim YC, Wikstrom M, Hummer G. Kinetic gating of the proton pump in cytochrome c oxidase. *Proc. Natl. Acad. Sci. U. S. A.* 2009; 106:13707–13712. [PubMed: 19666617]
- [34]. Muijsers AO, Tiesjema RH, Henderson RW, van Gelder BF. Biochemical and biophysical studies on cytochrome aa3. VII. The effect of cytochrome c on the oxidation-reduction potential of isolated cytochrome aa3. *Biochim. Biophys. Acta.* 1972; 267:216–221. [PubMed: 4336312]
- [35]. Tiesjema RH, Muijsers AO, van Gelder BF. Biochemical and biophysical studies on cytochrome c oxidase. X. Spectral and potentiometric properties of the hemes and coppers. *Biochim. Biophys. Acta.* 1973; 305:19–28. [PubMed: 4352553]
- [36]. Safwat HH. Effect of centrally located samples in the integrating sphere. *J. Opt. Soc. Am.* 1970; 60:534–541.
- [37]. Nelson NB, Prezelin BB. Calibration of an integrating sphere for determining the absorption coefficient of scattering suspensions. *Appl. Opt.* 1993; 32:6710–6717. [PubMed: 20856523]
- [38]. French SA, Territo PR, Balaban RS. Correction for inner filter effects in turbid samples: fluorescence assays of mitochondrial NADH. *Am. J. Physiol.* 1998; 275:C900–C909. [PubMed: 9730975]
- [39]. Heineman FW, Eng J, Berkowitz BA, Balaban RS. NMR spectral analysis of kinetic data using natural lineshapes. *Magn Reson. Med.* 1990; 13:490–497. [PubMed: 2325549]
- [40]. Wikström M, Morgan JE. The dioxygen cycle. Spectral, kinetic, and thermodynamic characteristics of ferryl and peroxy intermediates observed by reversal of the cytochrome oxidase reaction. *J. Biol. Chem.* 1992; 267:10266–10273. [PubMed: 1316895]
- [41]. Wikström M. Energy-dependent reversal of the cytochrome oxidase reaction. *Proc. Natl. Acad. Sci. U. S. A.* 1981; 78:4051–4054. [PubMed: 6270657]
- [42]. Bose S, French S, Evans FJ, Joubert F, Balaban RS. Metabolic network control of oxidative phosphorylation: multiple roles of inorganic phosphate. *J. Biol. Chem.* 2003; 278:39155–39165. [PubMed: 12871940]
- [43]. Verkhovsky MI, Morgan JE, Wikström M. Redox transitions between oxygen intermediates in cytochrome-c oxidase. *Proc. Natl. Acad. Sci. U.S.A.* 1996; 93:12235–12239. [PubMed: 8901563]
- [44]. Wikström KF, Harmon HJ, Ingledew WJ, Chance B. A re-evaluation of the spectral, potentiometric and energy-linked properties of cytochrome c oxidase in mitochondria. *FEBS Lett.* 1976; 65:259–277. [PubMed: 182532]

- [45]. Kim N, Ripple MO, Springett R. Spectral components of the alpha-band of cytochrome oxidase. *Biochim. Biophys. Acta*. 2011; 1807:779–787. [PubMed: 21420929]
- [46]. Mason MG, Nicholls P, Cooper CE. The steady-state mechanism of cytochrome c oxidase: redox interactions between metal centres. *Biochem. J*. 2009; 422:237–246. [PubMed: 19534725]
- [47]. Balaban RS, Bose S, French S, Territo PR. Work-related cytosolic signaling between sarcoplasmic reticulum and mitochondria: role of Ca^{2+} . *Mol. Biol. Cell*. 2001; 12:2101.
- [48]. Henry PD, Schuchleib R, Davis J, Weiss ES, Sobel BE. Myocardial contracture and accumulation of mitochondrial calcium in ischemic rabbit heart. *Am. J. Physiol*. 1977; 233:H677–H684. [PubMed: 596465]
- [49]. Stamm C, Friehs I, Cowan DB, Cao-Danh H, Noria S, Munakata M, McGowan FX Jr, Del Nido PJ. Post-ischemic PKC inhibition impairs myocardial calcium handling and increases contractile protein calcium sensitivity. *Cardiovasc. Res*. 2001; 51:108–121. [PubMed: 11399253]
- [50]. Borutaite V, Mildaziene V, Brown GC, Brand MD. Control and kinetic analysis of ischemia-damaged heart mitochondria: which parts of the oxidative phosphorylation system are affected by ischemia? *Biochim. Biophys. Acta*. 1995; 1272:154–158. [PubMed: 8541346]
- [51]. Bush LR, Shlafer M, Haack DW, Lucchesi BR. Time-dependent changes in canine cardiac mitochondrial function and ultrastructure resulting from coronary occlusion and reperfusion. *Basic Res. Cardiol*. 1980; 75:555–571. [PubMed: 7436999]
- [52]. Onsager L. Reciprocal relations in irreversible processes. I. *Phys. Rev*. 1931; 37:405–426.
- [53]. Onsager L. Reciprocal relations in irreversible processes. II. *Phys. Rev*. 1931; 38:2265–2279.
- [54]. Bloch D, Belevich I, Jasaitis A, Ribacka C, Pusstinen A, Verkhovsky MI, Wikstrom M. The catalytic cycle of cytochrome oxidase is not the sum of its two halves. *Proc. Natl. Acad. Sci. U.S.A.* 2004; 101:529–533. [PubMed: 14699047]
- [55]. Wikström M. Identification of the electron transfers in cytochrome oxidase that are coupled to proton-pumping. *Nature*. 1989; 338:776–778. [PubMed: 2469960]
- [56]. Lesnefsky EJ, Chen Q, Slabe TJ, Stoll MS, Minkler PE, Hassan MO, Tandler B, Hoppel CL. Ischemia, rather than reperfusion, inhibits respiration through cytochrome oxidase in the isolated, perfused rabbit heart: role of cardiolipin. *Am. J. Physiol Heart Circ. Physiol*. 2004; 287:H258–H267. [PubMed: 14988071]
- [57]. Rouslin W, Ranganathan S. Impaired function of mitochondrial electron transfer complex I in canine myocardial ischemia: loss of flavin mononucleotide. *J. Mol. Cell Cardiol*. 1983; 15:537–542. [PubMed: 6231381]
- [58]. Borutaite V, Morkuniene R, Budriunaite A, Krasauskaite D, Ryselis S, Toleikis A, Brown GC. Kinetic analysis of changes in activity of heart mitochondrial oxidative phosphorylation system induced by ischemia. *J. Mol. Cell Cardiol*. 1996; 28:2195–2201. [PubMed: 8930814]
- [59]. Lesnefsky EJ, Moghaddas S, Tandler B, Kerner J, Hoppel CL. Mitochondrial dysfunction in cardiac disease: ischemia-reperfusion, again, and heart failure. *J. Mol. Cell. Cardiol*. 2001; 33:1065–1089. [PubMed: 11444914]
- [60]. Chen Q, Lesnefsky EJ. Depletion of cardiolipin and cytochrome *c* during ischemia increases hydrogen peroxide production from the electron transport chain. *Free Rad. Biol. Med*. 2006; 40:976–982. [PubMed: 16540393]
- [61]. Chen Q, Moghaddas S, Hoppel CL, Lesnefsky EJ. Ischemic defects in the electron transport chain increase the production of reactive oxygen species from isolated rat heart mitochondria. *Am. J. Physiol. Cell Physiol*. 2008; 294:460–466.
- [62]. Paillard M, Gomez L, L Augeul, J Loufouat, Lesnefsky EJ, Ovize M. Postconditioning inhibits mPTP opening independent of oxidative phosphorylation and membrane potential. *J. Mol. Cell. Cardiol*. 2009; 46:902–909. [PubMed: 19254723]
- [63]. Chen Q, Yin G, Stewart S, Hu Y, Lesnefsky EJ. Isolating the segment of the mitochondrial electron transport chain responsible for mitochondrial damage during cardiac ischemia. *Biochem. Biophys. Res. Comm*. 2010; 397:656–660. [PubMed: 20529665]
- [64]. Holmuhamedov EL, Oberlin A, Short K, Terzic A, Jahangir A. Cardiac subsarcolemmal and interfibrillar mitochondria display distinct responsiveness to protection by diazoxide. *PLoS One*. 2012; 7:1–7.

- [65]. Kurian GA, Berenshtein E, Saada A, Chevion M. Rat cardiac mitochondrial subpopulations show distinct features of oxidative phosphorylation during ischemia, reperfusion, and ischemic preconditioning. *Cell. Physiol. Biochem.* 2012; 30:83–94. [PubMed: 22759958]
- [66]. Glancy B, Balaban RS. Role of mitochondrial Ca^{2+} in the regulation of cellular energetics. *Biochemistry.* 2012; 51:2959–2973. [PubMed: 22443365]
- [67]. Heineman FW, Kupriyanov VV, Marshall R, Fralix TA, Balaban RS. Myocardial oxygenation in the isolated working rabbit heart as a function of work. *Am. J. Physiol.* 1992; 262:255–267.

Figure 1. The integrating sphere system

(A) Photograph with red light impinging on the sample for visualization. The ports are present on the equator of the sphere. Attached to the port on the far left is the optical fiber delivering incident light. The light is collimated (Ocean Optics 74-UV) to contain the incident light on the sample. The reflective cap for the center port has been removed to allow visualization of the collimated incident beam striking the center-mounted cuvette and for experimental setup. This is replaced during experiments. The far-right port permits access of an afferent water line, which is connected to a peripheral water bath to maintain temperature at 37° C. The tubing has been covered with black foil to prevent room light from entering the sphere. During experiments, the whole system was covered with a black tarp to further obstruct room light. (B) Schematic diagram of the internal orientation of the sphere (note the position of the detector and injection ports). During experiments, substrate additions were made through the injection port created in the top of the sphere. The magnetic stirrer rested on the base of the cuvette holder and was attached to an external control unit by a flexible wire (not shown). Stirring was held constant at 1200 rpm during mitochondrial respiration experiments. Mixing time in this cylindrical chamber was evaluated with injected dyes and reached equilibrium in under 200 ms.

Figure 2. Absorbance difference spectra of pig heart mitochondria (1 nmol cytochrome aa_3 /mL) under different experimental conditions

(A) Absolute oxidized and reduced spectra of isolated mitochondria as measured in a conventional split-beam spectrometer (Perkin Elmer). Due to scattering and the distance between the cuvette and photomultiplier tube, there are high absorbance values and low signal-to-noise in the difference spectrum (B) due to lack of scattered light detection. (C) Absolute oxidized and reduced spectra of isolated mitochondria measured in a conventional spectrometer but solubilized in Triton X-100. Solubilization removes the scattering term, thereby lowering the “apparent” absorbance and drastically increasing the signal-to-noise of the difference spectrum (D). Peaks for a number of mitochondrial chromophores (*FN* and

*aa*₃, *b*, and *c*) are labeled. (E) Absolute oxidized and reduced spectra of isolated mitochondria suspended in respiration medium acquired using the integrating sphere. Here, even under highly-scattering conditions, signal-to-noise and spectral resolution are comparable to solubilized mitochondria due to collection of scattered photons by the sphere. (F) Reduced (CR) minus oxidized difference spectrum of data presented in (E).

Figure 3. Experimental protocol and spectral fitting

(A) Initially, an oxidized state was created by adding P_1 (3 mM) and occasionally a small amount of ADP (50 to 100 μ M) in the absence of carbon substrates (the O condition). This was followed by the addition of 5 mM glutamate and malate to substrate reduce the sample (SR). Each of these periods usually ran for 1 to 3 minutes. To initiate ATP synthesis, a 500 μ M or higher concentration of ADP was injected into the chamber (ATPs). This was followed by anoxia and/or the addition of sodium hydrosulfite to chemically reduce the sample (CR). (B) O to SR transition difference spectra. The panel on the left has the raw data (red line), fit data (blue line) and the residuals of the fit (green line). (C) The individual spectra used to fit the data. This scheme is the same for all of the subsequent panels in this

figure. (D) SR to ATPs transition difference spectra and associated spectral components (E). (F) O to CR transition difference spectra and associated spectral components (G).

Figure 4. Effect of incremental increases in ATP hydrolysis

(A) Difference spectra from the initial ATP only condition to the varying levels of apyrase added to the chamber. The increases in apyrase were titrated to create roughly equal step increases in ATP hydrolysis. The inset plots the J_0 versus $\Delta\Psi$ for these studies. (B) Plot of J_0 versus a607. (C) Plot of $\Delta\Psi$ versus a607. (D) Plot of J_0 versus a607 using variable amounts of apyrase (solid symbols) or FCCP (open symbols). (E) Plot of J_0 versus a580. (F) Plot of $\Delta\Psi$ versus a580. Both a607 and a580 are expressed as relative content as these are transient intermediates and do not exist in the fully oxidized or fully reduced state.

Table 1
Summary of mitochondrial chromophore redox states or relative contents during respiratory transitions

Condition	<i>FN</i>	<i>b_L</i>	<i>b_H</i>	<i>c_I</i>	<i>c</i>	<i>a580^b</i>	<i>a607^b</i>
G/M [SR]	100 ^a	100 ^a	29.0 ± 2.0	17.0 ± 1.2	13.0 ± 0.5	100 ^a	3.0 ± 0.2
ADP [ATPs]	32 ± 2.5	27 ± 1.3	28.0 ± 1.7 ^c	20.0 ± 0.8	17 ± 0.7	23 ± 2.6	7.0 ± 0.7

Data are means ± S.E.M., *n* = 11 per group.

^a Assumed 100% reduced under the GM condition

^b Expressed as relative contents as these are transient intermediates and do not exist in the fully oxidized or reduced states

^c Cytochrome *b_H* only species not to show a significant difference from GM to ATPs transition (*p* > 0.05)

Table 2
Calculated apparent conductances of oxidative phosphorylation reactions in rabbit heart mitochondria

J_o (nmol O_2 /min/nmol cyt aa ₃)		$\Delta\Psi$ (mV)	ΔG (kcal/mol)		Conductance			
Control	I/R	Control	I/R	Electron Transfer Step	Calculated ΔG (kcal/mol)		$Conductance = mVO_2 \div \Delta G $	
					Control	I/R	Control	p
256.00 $\pm$ 7.83	169.50 $\pm$ 16.87* ($p = 3.0 \times 10^{-4}$)	-147.06 $\pm$ 0.89	-144.84 $\pm$ 1.52	NAD ⁺ /NADH – Cyt c (– $\Delta\Psi$)	-6.10 $\pm$ 0.25	-6.67 $\pm$ 0.34	42.50 $\pm$ 3.28	20.45 $\pm$ 2.08*
				Cyt c – Cyt a	-3.65 $\pm$ 0.10	-3.59 $\pm$ 0.13	71.53 $\pm$ 3.25	37.40 $\pm$ 2.55*
				Cyt c – O ₂ /H ₂ O (– $\Delta\Psi$)	-12.43 $\pm$ 0.11	-12.70 $\pm$ 0.22	20.88 $\pm$ 0.85	10.47 $\pm$ 0.70*
				Cyt a – O ₂ /H ₂ O (– $\Delta\Psi$)	-8.78 $\pm$ 0.19	-9.08 $\pm$ 0.19	29.64 $\pm$ 1.32	14.63 $\pm$ 1.07*
				NAD ⁺ /NADH – O ₂ /H ₂ O (– $\Delta\Psi$)	-18.42 $\pm$ 0.25	-19.28 $\pm$ 0.52	13.71 $\pm$ 0.73	6.90 $\pm$ 0.53*

Data are means $\pm$ S.E.M., $n = 4$ per group.