Regulation of oxidative phosphorylation complex activity: effects of tissue-specific metabolic stress within an allometric series and acute changes in workload

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Phillips D, Covian R, Aponte AM, Glancy B, Taylor JF, Chess D, Balaban RS. Regulation of oxidative phosphorylation complex activity: effects of tissue-specific metabolic stress within an allometric series and acute changes in workload. Am J Physiol Regul Integr Comp Physiol 302: R1034–R1048, 2012. First published February 29, 2012; doi:10.1152/ajpregu.00596.2011.—The concentration of mitochondrial oxidative phosphorylation complexes (MOPCs) is tuned to the maximum energy conversion requirements of a given tissue; however, whether the activity of MOPCs is altered in response to acute changes in energy conversion demand is unclear. We hypothesized that MOPCs activity is modulated by tissue metabolic stress to maintain the energy-metabolism homeostasis. Metabolic stress was defined as the observed energy conversion rate/maximum energy conversion rate. The maximum energy conversion rate was assumed to be proportional to the concentration of MOPCs, as determined with optical spectroscopy, gel electrophoresis, and mass spectrometry. The resting metabolic stress of the heart and liver across the range of resting metabolic rates within an allometric series (mouse, rabbit, and pig) was determined from MOPCs content and literature respiratory values. The metabolic stress of the liver was high and nearly constant across the allometric series due to the proportional increase in MOPCs content with resting metabolic rate. In contrast, the MOPCs content of the heart was essentially constant in the allometric series, resulting in an increasing metabolic stress with decreasing animal size. The MOPCs activity was determined in native gels, with an emphasis on Complex V. Extracted MOPCs enzyme activity was proportional to resting metabolic stress across tissues and species. Complex V activity was also shown to be acutely modulated by changes in metabolic stress in the heart, in vivo and in vitro. The modulation of extracted MOPCs activity suggests that persistent posttranslational modifications (PTMs) alter MOPCs activity both chronically and acutely, specifically in the heart. Protein phosphorylation of Complex V was correlated with activity inhibition under several conditions, suggesting that protein phosphorylation may contribute to activity modulation with energy metabolic stress. These data are consistent with the notion that metabolic stress modulates MOPCs activity in the heart.

MITOCHONDRIA are dynamic intracellular organelles that play a major role in eukaryotic cellular energy conversion, intermediary metabolism, calcium homeostasis, cell signaling, and apoptosis. With regard to energy conversion, mitochondria are capable of precisely orchestrating the generation of ATP to match the ATP hydrolysis required to perform cellular work. In recent years an increasing body of evidence has revealed that the expression level of mitochondrial proteins is finely tuned to the functional requirements of different tissues and disease states (11, 27, 28, 38–40, 49, 60, 63, 72, 89). Notably, these differences in “protein programming” are realized by regulating most, if not all, of the enzymes in a given metabolic pathway, as opposed to individual rate-limited steps (38). Consistent with this notion, the mitochondrial oxidative phosphorylation complexes (MOPCs) responsible for the generation of ATP are upregulated in tissues with high-energy demands, such as the heart compared with liver (11, 38). Thus adjusting the expression level of MOPCs across different tissues provides a long-term regulatory mechanism for matching maximum mitochondrial ATP production with the maximum work requirements of a given tissue (11, 40, 78). This principle of matching the expression level of metabolic enzymes with the maximum demands of a tissue was first outlined by Weibel and coworkers (87) and termed symmetry. While the relative concentration of MOPCs has been shown to be proportional to the maximum metabolic rate across tissues, the mechanisms involved in altering the activity of MOPCs to dynamically modulate ATP production in concert with ATP hydrolysis remains elusive.

The regulation of MOPCs activity can be accomplished by several means. The simplest mechanism involves the feedback of substrates for an enzymatic reaction, such as ADP and P_i, for the ATP synthetic reaction. However, it has been shown in several systems, most notably the heart (see reviews Refs. 6 and 7) and to a lesser extent skeletal muscle (33), that large changes in mitochondrial synthetic rate are not associated with significant alterations in the substrates for ATP production or the net free energy in cytosolic ATP (ΔG_ATP). This ability to maintain a near-constant ΔG_ATP with changes in workload has been termed metabolic homeostasis (5, 33). Thus simple substrate feedback does not seem adequate to coordinate mitochondrial ATP synthesis with work requirements.

Another mechanism for modulating enzyme function involves covalent posttranslational modifications (PTMs), which can alter activity with little or no change in reaction substrates. Given the observed metabolic homeostasis that occurs during alterations in workload, PTMs are the best candidate for acutely regulating MOPCs enzyme activity to meet increases in ATP demand. The classic example of this type of regulation is mitochondrial pyruvate dehydrogenase (PDH), which is inactivated by phosphorylation [i.e., effectively reduces the maxi-
Maximum velocity ($V_{\text{max}}$) of the reaction during times of low activity (18, 46, 70). $\text{Ca}^{2+}$ regulates PDH phosphorylation through modulation of PDH phosphatase I (24, 65). In addition, $\text{Ca}^{2+}$ also alters the affinity of isocitrate dehydrogenase (ICDH) and $\alpha$-ketoglutarate dehydrogenase ($\alpha$KDH) for carbon substrates through noncovalent allosteric interactions (20).

The question remains whether PTMs could play a wider role in the acute regulation of mitochondrial energy conversion. Recent studies have revealed numerous persistent PTMs on MOPCs, including acetylation (2, 10), phosphorylation (4, 37, 52, 77), nitrosylation (82), ADP-ribosylation (50), and oxidation (53). Additionally, several PTMs have been correlated with alteration in Complex I (17, 64, 79), Complex IV (1, 12, 52), and Complex V (76, 84) activities. This list of mitochondrial protein PTMs is growing rapidly with the development of sophisticated mass spectrometry methodologies; however, very few functional consequences of these interactions have been established. Thus, whereas a wide variety of PTMs are present in the MOPCs, the functional significance of these sites is poorly defined.

In the current study, we hypothesize that PTMs modulate the activity of MOPCs to allow a tissue to maintain a metabolic homeostasis in the face of persistent and acute changes in energy demand. This hypothesis addresses the metabolic activity “downstream” of the citric acid cycle reactions discussed above. We proposed that this coordinated system of PTMs would be more prevalent in tissues and animals with large dynamic ranges in ATP generation compared with those systems operating near their maximum capacity. Consistent with these regulators being reversible PTMs, we reasoned that they would persist in isolated MOPC activity assays, as was originally demonstrated for PDH (22). To test this, we screened for regulatory PTMs using the activity of extracted MOPCs. We defined MOPC activity as the catalytic turnover rate per mole of complex. To estimate energy demand, we calculated metabolic stress [i.e., energy conversion rate/maximum energy conversion rate (29)] where the maximum energy conversion rate was assumed to be proportional to total MOPC content.

We evaluated our hypothesis using three systems. First, we focused on “resting” conditions in the pig heart and liver. These tissues were selected because their MOPCs have only minor differences in protein sequence but possess very different dynamic ranges in ATP production. For instance, the large animal heart has a dynamic range of ATP production that exceeds 10-fold (59, 86), whereas the liver has a dynamic range of less than 2-fold (45, 54, 80). Second, MOPC activity was determined in an allometric series (i.e., pig, rabbit, and mouse) to take advantage of the heart’s decreasing resting metabolic rate and increasing dynamic range of energy metabolism with increasing body size. Third, we acutely altered the workload of the pig heart in vivo and the rabbit heart in vitro to determine whether the persistent inhibition of MOPC activity in the “resting” heart could be removed by an increase in workload.

In this study we demonstrate that MOPC activity parallels metabolic stress. We show that MOPC activity is persistently inhibited at “rest” in animals and tissues with large dynamic ranges of ATP production, with the ability to release this inhibition during increased workload. Finally, the in vitro activity of Complex V was inversely correlated with $\gamma$-subunit protein phosphorylation consistent with a role of protein phosphorylation in the acute modulation of Complex V activity and potentially the maintenance of the metabolic homeostasis in heart.

**MATERIALS AND METHODS**

**Materials.** Salts and P, were purchased from Sigma (St. Louis, MO). Two-dimensional (2D) gel electrophoresis reagents CyDyes, equipment, and software were purchased from GE Healthcare (Piscataway, NJ). Native PAGE buffers, reagents, and gels were purchased from Invitrogen (Carlsbad, CA). $32^P$ (10 mCi/ml) was purchased from PerkinElmer (Boston, MA).

**Mitochondrial isolation.** All procedures performed were in accordance with the Animal Care and Welfare Act (7 U.S.C. 2142 § 13) and approved by the NHLBI ACUC. Heart mitochondria were isolated from animals using an in situ cold perfusion to prevent warm ischemia and remove blood and extracellular $\text{Ca}^{2+}$ as described (30). One modification was that 1 mM K$_2$PO$_4$ was added to buffer A (0.28 M sucrose, 10 mM HEPES, 1 mM EDTA, and 1 mM EGTA; pH 7.1) for the first mitochondrial resuspension. This was done to avoid phosphate depletion of the mitochondria matrix, as described previously (4). Mitochondria were then washed twice with buffer A alone, once with buffer B (137 mM KCl, 10 mM HEPES, 2.5 mM MgCl$_2$, and 0.5 mM K$_2$EDTA), and finally resuspended in buffer B. Liver mitochondria were isolated from the same animals as the heart mitochondria following a similar protocol (4). After euthanasia the liver was immediately removed and flushed with 1 liter of cold buffer A to chill the tissue and remove blood. Only visually blanched tissue was used for isolation. After fat and connective tissue were removed, the liver was finely chopped in cold buffer A, homogenized twice with a loose-fitting tissue grinder, and centrifuged at 600 g for 10 min at 4°C. The mitochondrial pellet was washed and resuspended following the same protocol described above.

Mitochondrial preparations were tested for viability by measuring the respiratory control ratio (RCR), which involved taking the ratio of the rate of oxygen consumption in the presence and absence of ADP (1 mM) at 37°C with the following incubation medium: buffer B containing 5 mM potassium-glutamate, 5 mM potassium-malate, and 1 mM P$_i$. To accept a mitochondrial preparation, the RCR had to exceed 8 in the heart and 5 in the liver to assure a well-coupled system. The reasons for the lower RCR in liver are unknown.

In this study, isolated mitochondria were used for protein analysis only. Viability was evaluated to assure complexes isolated were from functional mitochondria. No attempt was made to correlate in vitro mitochondrial activity using State 3/4 respiration between organs or animals. The reasons for avoiding this type of comparison included the following: 1) Different carbon substrate oxidation capacities in different tissues (38). Thus direct comparisons are nearly impossible to make with a given reduced carbon source. 2) Alterations in the matrix milieu induced by the isolation process, such as $P_i$, $Ca^{2+}$, and volume, that could alter the distribution of PTMs from the in vivo state. The alteration in PTM with isolation was demonstrated in some of the original work on PDH (43) that showed a time-dependent alteration of PDH activity in isolated mitochondria. We have also found that isolated mitochondria complex activities are generally higher than assays performed on complexes rapidly isolated directly from tissue biopsies consistent with the isolation environments influencing the PTM profile of these preparations.

**Spectrophotometric cytochrome quantification.** Mitochondrial cytochrome a$_{33}$ (cyto a) content was assayed from an oxidized-reduced spectrum of a Triton X-100-treated mitochondrial suspension as described (30). One modification was that 1 mM K$_2$PO$_4$ was added to buffer A (0.28 M sucrose, 10 mM HEPES, 1 mM EDTA, and 1 mM EGTA; pH 7.1) for the first mitochondrial resuspension. This was done to avoid phosphate depletion of the mitochondria matrix, as described previously (4). Mitochondria were then washed twice with buffer A alone, once with buffer B (137 mM KCl, 10 mM HEPES, 2.5 mM MgCl$_2$, and 0.5 mM K$_2$EDTA), and finally resuspended in buffer B. Liver mitochondria were isolated from the same animals as the heart mitochondria following a similar protocol (4). After euthanasia the liver was immediately removed and flushed with 1 liter of cold buffer A to chill the tissue and remove blood. Only visually blanched tissue was used for isolation. After fat and connective tissue were removed, the liver was finely chopped in cold buffer A, homogenized twice with a loose-fitting tissue grinder, and centrifuged at 600 g for 10 min at 4°C. The mitochondrial pellet was washed and resuspended following the same protocol described above.

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**Spectrophotometric cytochrome quantification.** Mitochondrial cytochrome a$_{33}$ (cyto a) content was assayed from an oxidized-reduced spectrum of a Triton X-100-treated mitochondrial suspension as previously described (9). Briefly, Triton X-100-treated extracts were assayed at 605 nm (with reference created from a linear regression between 575 and 630 nm) using cyanide to generate a fully reduced spectrum in the presence of blood and myoglobin. The cyto a content was determined using an extinction coefficient of 10.8 mM$^{-1}$ cm$^{-1}$ with cyanide as a reducing agent and 12 mM$^{-1}$ cm$^{-1}$ when sodium...
hydrosulphite was used in mitochondria extracts. Because of the interferences from hemoglobin and myoglobin, cyto c and b were not determined in whole tissue extracts using this approach.

The cytochrome measurements were used to determine the content of oxidative phosphorylation enzymes in different tissues and species. Cyto c measurements were reported as 10⁻¹⁵ moles (nmole) of cyto c per mg mitochondrial protein or gram of wet heart weight. Since the mitochondria of the heart and liver vary considerably in their composition (i.e., heart mitochondria per milligram of mitochondrial protein contain more oxidative phosphorylation enzymes, whereas liver mitochondria contain more urea cycle enzymes), we used cyto c to match oxidative phosphorylation enzyme content between tissues. Native in-gel activity assays were loaded per cyto c a content and further normalized per mole of MOPC based on Coomassie blue staining (see Complex activity measurements).

**32P-labeling of intact mitochondria.** 32P-labeling of intact mitochondria and isolated Complex V was performed as previously described (4). Briefly, intact pig heart and liver mitochondria were incubated at 37°C for 20 min in oxygenated Buffer C (125 mM KCl, 15 mM NaCl, 20 mM HEPES, 1 mM EGTA, 1 mM EDTA, 5 mM MgCl₂, 5 mM potassium-glutamate, and 5 mM potassium-malate; pH 7.1) and 250 μCi/mg of 32P. Complex V was then isolated using an immunoprecipitation kit (Mitosciences, Eugene, OR), according to the manufacturer’s instructions. Forty micrograms of purified 32P-labeled Complex V from each tissue were then analyzed by 2D gel electrophoresis as described below.

**2D gel electrophoresis and analysis.** 2D-labeled Complex V and CyDye-labeled heart and liver mitochondrial proteins were analyzed by 2D gel electrophoresis, as previously described (4). Proteins were separated first by charge via 24-cm IPG strips (pH 3–10 NL) and then by mass on 10–15% gradient gels. 32P-labeled protein gels were stained with Coomassie blue, dried, exposed to a phosphor screen, and imaged; CyDye-labeled gels were imaged immediately following electrophoresis, as described previously (4). To identify proteins from the 2D gels, paired nondialyzed gels were picked using the Ettan Spot Handling Workstation (GE Healthcare) and identified using a MALDI-TOF/TOF instrument (4700 Proteomic Analyser, Applied Biosystems), as previously described (4).

The differential in-gel electrophoresis (2D-DIGE) was also used to estimate absolute protein concentrations in the different tissues, using the relative areas of the peaks and the molecular weights versus proteins of known concentration. For these studies we used the absolute concentration of Complex IV (cyto a), determined spectrophotometrically, as a reference for quantization of other proteins in the 2D-DIGE gel.

**Isobaric tags for relative and absolute quantitation processing.** To obtain more comprehensive protein coverage and relative protein concentrations as well as information on subunit composition, a mass spectrometry study using isobaric tags for relative and absolute quantitation (iTRAQ)-labeling was performed on heart and liver mitochondria from the pig and mouse. The iTRAQ approach overcomes the quantitative limitation of label-free techniques (39). One hundred micrograms of acetone-precipitated protein lysate was iTRAQ-labeled according to the manufacturer’s instructions (Invitrogen). Isobaric tagging was performed from mass-to-charge (m/z) 113–116 for the mouse heart samples and m/z 117–121 for the mouse liver samples. The pig heart samples were labeled with tags m/z 114–116 and liver samples with m/z 118–121. All iTRAQ data for mouse and pig data sets were presented as the ratio of heart proteins to liver proteins. Both pig and mouse sets were prepared separately but followed the same methods throughout. After quenching the labeling reaction, the resulting peptide mixtures were combined and dried until the final volume of 100 μl was achieved. The combined peptide digest was resuspended in 900 μl of 0.1% formic acid and desalted using Waters Oasis HLB 1-cm³ cartridges (Milford, MA) per the manufacturer’s instructions using acetonitrile instead of methanol.

Eluent was dried and resuspended with 100 μl strong cation exchange chromatography buffer A (10 mM KH₂PO₄/25% acetonitrile, pH 3.0).

**Strong cation exchange chromatography.** An Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) was used to separate the peptides by their charge using a polysulfonyl A column; 200 × 2.1 mm, particle size 5 μm, 200 A (Poly LC, Columbia, MD). A 60-min linear ramp from 0% to 40% buffer B (10 mM KH₂PO₄, 500 mM KCl-25% ACN, pH 2.7) was used to separate the peptides. Column temperature was maintained at room temperature, and a flow rate of 200 μl/min was maintained throughout the run. Fractions were collected at 1-min intervals on a 96-well microtiter plate for a total of 60 fractions. The chromatographic peaks were monitored using the built in UV detector (214 nm), and fractions were combined for a final count of 23 fractions. Each fraction was desalted using Waters Oasis HLB 1 cm³ cartridges (Milford, MA) per the manufacturer’s instructions using acetonitrile instead of methanol.

**Mass spectrometry LC MS/MS: orbitrap velos.** Liquid chromatography tandem mass spectrometry was performed using an Eksigent nanoLC-Ultra 1D plus system (Dublin, CA) coupled to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The peptide digest of 10 μl was first loaded onto an Zorbax 300SB-C18 trap column (Agilent, Palo Alto, CA) at a flow rate of 6 μl/min for 6 min and then separated on a reversed-phase PicoFrit analytical column (New Objective, Woburn, MA) using a 40-min linear gradient of 5–40% acetonitrile in 0.1% formic acid at a flow rate of 250 nl/min. LTQ-Orbitrap Velos settings were as follows: spray voltage 1.5 kV; full MS mass range: m/z 300 to 2,000, operated in positive ion mode with data-dependent acquisition. A single full-scan MS in the Orbitrap (30,000 resolution, 300–2,000 m/z) was followed by six data-dependent MS2 scans for precursor ions above a threshold ion count of 10,000, using the HCD cell with the resolution set to 7,500 and 45% normal collision energy.

**Mass spectrometry data analysis.** The raw files generated were analyzed for protein identification and quantification using Proteome Discoverer v1.2 software (Thermo Fisher Scientific, San Jose, CA) using Mascot search engine v.2.3 (http://biospec.nih.gov) managed and weekly updated protein databases by the Center for Information Technology at the NIH. The MS/MS spectra were searched against the Swiss-Prot Protein Knowledgebase (Sprot) combined pig, bovine, and human databases (release 201105). The combined pig, bovine, and human database was used because the pig protein database is partially annotated. For the mouse samples, the taxonomy was set to house mouse. The enzyme parameters were limited to trypsin with a maximum miss cleavages set to 2. The selection criteria were the following: variable modifications; oxidation (M), deamidation (NQ), iTRAQ 8plex (Y); fixed modifications, (MMTS) methyl methanethiosulfonate (C), NH₂-terminal iTRAQ 8plex, iTRAQ8plex (K); 10 ppm for precursor ions and 0.05 Da for fragment ions. The quantitative measurements were performed in the MS/MS scan using the iTRAQ reporter ions. Software normalization was not performed. The automatic decoy database search option was selected on Mascot, and the high confidence peptides were only used for protein identification that contained a false discovery rate (FDR) <1% at the peptide level. Briefly, every time a peptide sequence search is performed on a target database, a random sequence of equal length is automatically generated and tested. The statistics for matches are calculated and a peptide significance is generated, and an in-depth explanation can be found www.matrixscience.com. Protein identifications with two or more unique peptides were selected for quantitative analysis. All mass spectra used in this study are publicly available at the Proteome Commons Tranche web site (https://proteomecommons.org/group.jsp?i=219).

**Complex activity measurements.** The activities of MOPCs were determined with various assays within Blue Native polyacrylamide gel electrophoresis (BN-PAGE). BN-PAGE gels were used to isolate and measure Complex I, Complex IV, and Complex V activity in isolated pig heart and liver mitochondria; in vivo biopsy samples from pig heart and liver; and whole tissue extracts from pig, rabbit, and...
murine heart and liver. Since heart and liver have different amounts of MOPC per milligram of mitochondria, cyto a measurements (obtained spectrophotometrically) were used to normalize MOPC content between tissues. Cyto a (Complex IV) was used as a reference because its ratio to Complexes I and V was constant based on the 2D-DIGE gels. This finding was confirmed by comparing the ratio of Coomassie staining intensity for Complexes I and V to Complex IV in BN-PAGE, which was essentially identical to using cyto a as a reference for the protein loading.

BN-PAGE relies on the binding of Coomassie blue to mitochondrial protein complexes to enhance their differential migration during the PAGE process (74). For isolated mitochondria, BN-PAGE was performed on mitochondrial pellets according to the Invitrogen protocol for the Native PAGE Novex Bis-Tris Gel System, with dodecyl maltside as the detergent. Since liver contains fewer moles of MOPC per milligram of mitochondrial protein, isolated mitochondrial pellets were solubilized per milligram (to maintain a correct dodecyl-maltside-to-protein ratio) and then loaded per picomole of cytochrome a (to ensure matched cytochrome content). Four-16% 1-mm bis-Tris gels were used, and 50 pmol of mitochondrial protein were loaded per lane. BN-PAGE was performed at 4°C for 1 h at 150 V and then for 1.3 h at 250 V. Gels used for Complex V activity assays remained in dark cathode buffer for the entire run, whereas gels used for Complex I and Complex IV activity assays switched to light cathode buffer after the first hour of electrophoresis.

After electrophoresis, BN-PAGE gels were photographed to accurately quantify the Coomassie signal (i.e., protein content), and activity assays were then performed. Assays were performed using literature procedures for Complexes I and IV (44) and Complex V (14) with several modifications. For the Complex I activity assay, 0.75 g of nitrotetrazolium blue (NBT) were used and the assay was allowed to proceed for 5 min. For the Complex IV activity assay, 0.01 g of cytochrome c from bovine was used and the assay was allowed to proceed for 10 min. For the Complex V activity assay, the assay buffers were adjusted to a pH of 7.8, and the incubation steps were as follows: preincubation buffer (35 mM Tris-HCl, 270 mM glycine, 14 mM MgSO₄, pH 7.8) for 20 min and incubation buffer [35 mM Tris-HCl, 270 mM glycine, 14 mM MgSO₄, 0.2% (wt/vol) Pb(NO₃)₂, 8 mM ATP, pH 7.8] for 20 min. Great care was taken to avoid saturation. This was especially problematic for liver and mouse heart samples, which were relatively the most active. All gels were photographed immediately after completion of the activity assay. Complex I and Complex IV gels were photographed on a light box with no filtering. Complex V gels were placed on a flat flask containing a saturated solution of Coomassie Blue dye; this optimized the contrast of the scattered light from the reaction products. These gels were then photographed using an infrared light and Wratten filter (Kodak, Rochester, NY) to avoid the absorbance by Coomassie. Activities are reported as ratios or relative to the control condition, based on a given gel.

For in vivo biopsy samples or whole tissue homogenates, ~0.1 g of freshly perfused tissue (trimmed of fat and connective tissue) was added to 1 ml of buffer A, minced into small pieces, and homogenized with a Virtishear for 5 s for heart and 1 s for liver tissue on ice. Samples were then centrifuged at 10,000 g for 5 min at 4°C. supernatants were removed, and pellets were resuspended in 500 ml of buffer B. This step was repeated, and 1 nanomole of cyto a from the suspension was used for BN-PAGE activity assays.

Given differences in the Native PAGE modalities, clear native (CN)-PAGE gels were also run to confirm BN-PAGE results and utilize the oligomycin-sensitivity feature of the CN-PAGE (90). We preferred to use BN-PAGE because protein content and activity could be obtained from the same gel. When measuring activity via CN-PAGE, two gels were required: one gel was used to measure activity and a paired gel was stained with Coomassie blue and used to determine protein concentration. CN-PAGE samples were processed in the same manner as described for BN-PAGE above, except no Coomassie blue additive was included. Instead, an equivalent volume of 4× Native PAGE buffer was used. High-resolution CN-PAGE electrophoresis buffers were chosen according to previous studies (90). To assess Complex I and Complex V activity by CN-PAGE, the electrophoresis buffers used were the following: anode buffer (25 mM imidazole-HCl, pH 7.0) and cathode buffer 1 (50 mM Tricine, 7.5 mM imidazole, 0.02% dodecyl maltoside, 0.05% deoxycholate, pH 7.0). To assess Complex IV activity by CN-PAGE, the buffers used were the following: anode buffer (same as above) and cathode buffer 2 (50 mM Tricine, 7.5 mM imidazole, 0.05% deoxycholate, 0.05% Triton X-100, pH 7.0). CN-PAGE was run on 4–16% bis-Tris gels (Invitrogen), and samples were loaded as described above for BN-PAGE. CN-PAGE electrophoresis was conducted at 4°C at 150 V for 1 h, followed by 250 V for 1.4 h. Activity assays were performed as described for BN-PAGE, with the notable exception that Complex V showed oligomycin sensitivity when assayed with CN-PAGE.

Because of the importance of these assays in the interpretation of these data, the complete characterization of Native PAGE assays is shown in Fig. 1.

In vivo biopsy studies in pig hearts. To obtain biopsy samples, pigs (~50 kg) were given intravenous injection (in mg/kg: 25 ketamine, 0.5 midazolam, and 0.01 glycopyrrolate) for sedation. This was followed by intubation with an 8-mm endotracheal tube and anesthesia with 2% Isoflurane and a mixture of oxygen and medical air 35%-65% (Siemen’s Kion Ventilator/Anesthesia machine). Blood gases, heart rate, and arterial blood pressure were continuously monitored. A midline sternotomy was performed to expose the tissues. The heart was supported by a pericardial cradle, directly exposing it for biopsy collection. Biopsies were collected from the heart by angling a tissue core biopsy system with a 12-gauge coaxial biopsy needle (Bard Magnum biopsy Systems, Tempe, AZ) along the long axis of the heart and penetrating roughly halfway through the ventricle wall. Liver biopsy samples were collected from the same lobe. Three biopsy samples (~0.5 g per biopsy) were obtained per organ, immediately placed in ice-cold buffer A (0.28 M sucrose, 10 mM HEPES, 1 mM EDTA, and 1 mM EGTA; pH 7.1), and processed for BN-PAGE analysis as described above.

In vivo biopsy samples were also obtained from pig hearts under different workload conditions, mediated by dobutamine infusion. Dobutamine is a β-sympathetic nerve receptor agonist commonly used to increase cardiac inotropy and to a lesser extent heart rate in anesthetized animals (85). The pig was prepared as described above, except biopsy lacerations were sutured to prevent further bleeding. For each experimental condition, three biopsies (~0.5 g per biopsy) were taken. The control condition was at rest (predobutamine). Dobutamine was then infused at a dose of 20 μg·kg⁻¹·min⁻¹, and biopsies were taken once a maximum heart rate and pressure were reached. For the current study, “maximum” is defined as the peak heart rates and pressures attained during constant monitoring. To assist with venous return to the heart, the pig’s feet were elevated considerably and weights were placed on the abdomen. Dobutamine infusion was then stopped, and 15 min after the pig’s heart rate and pressures returned to predobutamine levels, three additional biopsies were taken. All biopsy samples were taken from the same plane of the left ventricle, but to avoid damaged tissue, great care was exercised not to take biopsies from previously sampled regions. Biopsy samples were processed for BN-PAGE analysis as described above. Heart rates and blood pressures were monitored throughout this experiment to calculate the natriuretic product.

The transmural activity of Complex V across the left ventricle wall was determined to establish whether it is homogenous and how critical sampling with a biopsy needle would be with regards to depth. The heart was surgically removed and immediately flushed with ice-cold isolation buffer as described above. The left ventricle was sectioned off from the right ventricle, and a basal section of left ventricle free wall was uniformly cut out. This piece was then cut into sections of inner, mid, and outer weighing ~0.1 g. The inner, mid, and
outer tissue sections were then processed for the CN-PAGE Complex V activity assay, as reported above. A Bradford protein assay was completed to normalize each sample for protein content. No significant differences in Complex V activity was found as a function of transmural position (Fig. 2). Thus differential contributions of transmural tissue to the biopsy samples transmurally would not be a source of variability in Complex V activity.

Perfused rabbit heart studies. To confirm the in vivo dobutamine studies in the pig heart and obtain a larger physiological dynamic range, rabbit hearts were Langendorff-perfused in vitro as previously reported (32). Briefly, hearts were removed from the anesthetized animal and placed in ice-cold saline for transfer to the perfusion apparatus. The hearts were perfused at 37°C at a constant pressure of 100 mm Hg with a coronary flow of 50 ml/min. The perfusion medium was composed of (in mM) 115 NaCl, 4 KCl, 1.6 CaCl₂, 1.4 MgSO₄, 1 KH₂PO₄, 25 NaHCO₃, 5.6 glucose, and 3 Na L-lactate. The solution was continuously equilibrated with 95% O₂ and 5% CO₂ to maintain the pH at 7.4. Heart rate, perfusion pressure, and developed pressure were monitored continuously. Three conditions were examined as the following: 1) control perfusion (1.25 h); 2) 1 h control

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**Fig. 1.** Native PAGE activity assay characterization. **A:** blue native (BN)-PAGE Complex IV activity assay characterization. **B:** BN-PAGE Complex V activity assay characterization. **C:** clear native (CN)-PAGE Complex IV activity assay characterization. **D:** CN-PAGE Complex V activity assay characterization. For **A–D:** 1) Coomassie-stained native gel; 2) gel activity assay at 10, 20, 40, and 60 min; 3) a plot of activity relative to protein concentration; and 4) a plot of activity as a function of time for the 25-, 50-, and 75-μg lanes. **E:** CN-PAGE Complex V activity assay characterization with ethanol control (E1) or 50 μM oligomycin (E2). **Left**, paired Coomassie-stained CN-PAGE gel; **right**, Complex V activity data at 10, 20, 40, and 60 min.
perfusion followed by 0.25 h of dobutamine 3-titration to obtain an approximately threefold increase in heart rate; and) 1.17 h control perfusion, followed by 0.08 h of KCl arrest. For harvesting, the hearts were perfused with ice-cold saline while still on the perfusion apparatus. The left ventricle was then excised and trimmed of fat and connective tissue. Approximately 0.5 g of tissue was homogenized in 5 ml of buffer A, and samples were processed for analysis by Native-PAGE as described above.

RESULTS

Content of mitochondrial oxidative phosphorylation complexes in the heart and liver. To determine the molar activities of the MOPCs in the heart and liver, the concentration of each Complex had to be obtained. Three methods were utilized to accomplish this task: optical spectroscopy of cytochrome a,a3 (cyto a, which is an element of Complex IV), 2D-DIGE, and iTRAQ mass spectrometry. Since the protein extraction procedures for optical spectroscopy differed significantly from those used for 2D-DIGE and iTRAQ, these measures can be treated independently, free of systematic protein processing errors. We first applied these techniques to isolated heart and liver mitochondria from pig and mouse. With the use of optical spectroscopy, the absolute cyto a content was determined: in pig mitochondria, heart (nmol/mg mitochondrial protein) $0.9 \pm 0.1$ ($n = 4$), liver $0.2 \pm 0.03$ ($n = 4$); in murine mitochondria, heart $1.0 \pm 0.3$ ($n = 3$), liver $0.15 \pm 0.01$ ($n = 6$). The concentration of cyto a was significantly higher in the heart in both species, with a heart-to-liver ratio of 4.5 in pig and 6.7 in mouse. These data are consistent with the results of Benard et al. in the rat (11). Additionally, the relative concentration of Complexes I, II, III, IV, and V in heart and liver mitochondria from the pig and mouse were determined with 2D-DIGE and iTRAQ. Consistent with the cyto a measurements and previous studies (11, 27, 38, 40, 60, 63), the 2D-DIGE (Fig. 3, A–B) and iTRAQ (see online supplemental Table S1) studies showed that the concentration of MOPCs is higher in heart than liver mitochondria. The complete iTRAQ datasets for pig (online Supplemental Table S2) and mouse (online Supplemental Table S3) are provided. Table 1 presents a quantitative summary of the heart-to-liver ratios of MOPCs content in pig mitochondria using optical spectroscopy, 2D-DIGE, and iTRAQ. Good agreement was generally found between methodologies, with an exception being the heart-to-liver ratio of Complex I, which was shown to be ~40% lower with 2D-DIGE. Using 2D-DIGE to quantify relative protein concentrations is complicated by the fact that proteins frequently undergo posttranslational modifications, can be relatively low in abundance, and rarely exist discretely within a gel. The Complex I subunits are especially prone to these complications, which likely accounted for the lower heart-to-liver ratio that was determined with 2D-DIGE. A comparison of the pig and mouse data revealed nearly identical heart-to-liver ratios for MOPC content, with the exception of Complex II (i.e., succinate dehydrogenase and electron transfer flavoprotein), which was relatively lower in mouse (see online Supplemental Tables S2 and S3 and DISCUSSION). Thus these studies showed that MOPC content is higher in the heart than the liver and underline the notion that simply

![Fig. 2. Transmural Complex V activity. In 3 pigs, transmural tissue samples from the outer, middle, and inner regions of the left ventricle were assayed for Complex V activity. A: raw CN-PAGE data; B: quantitative data.](image)

![Fig. 3. Mouse (A) and pig (B) where heart proteins are labeled red and liver green. The β subunit of Complex V is shown as an inset, since its color saturated at the window level necessary to reveal a majority of proteins.](image)
using mitochondrial volume to compare the aerobic metabolic capacity between tissues is not reliable.

We next used optical spectroscopy to determine the absolute concentration of cyto a and estimate the metabolic stress in heart and liver tissue homogenates from the pig, rabbit, and mouse (Table 2). Similar to isolated mitochondria, the cyto a content in total tissue homogenates was significantly higher in the heart than liver for all species. Notably, while the cyto a content from the heart was nearly constant across species, the liver cyto a content decreased with increasing animal size. To calculate the metabolic stress across these tissues and species, the cyto a measurements were combined with resting respiratory rates obtained from the literature. For pig and rabbit tissues, the resting respiratory rates were obtained from in vivo data, whereas mouse data were extrapolated from in vitro preparations (specific references are provided in Table 2). Even though the cyto a content in the heart is similar between the mouse and pig, the mouse heart has an approximately seven-fold higher resting respiratory rate (69, 83), which translates to a higher cyto a catalytic rate and thus a higher resting metabolic stress. In contrast, the cyto a content in liver increases proportionally with the resting metabolic rate, which results in a generally higher and constant as a function of body size.

Persistent regulation of the resting activities of Complexes I, IV, and V: higher in the liver than the heart and scale inversely with animal size. To test the hypothesis that MOPCs activity is regulated to match the energy metabolic stress, the molar activity of Complexes I, IV, and V were determined using native gel assays (74). Since the heart and liver have different activities of Complexes I, IV, and V were determined using BN-PAGE. The dobutamine-inhibitory PTMs associated with these MOPCs are labile, similar to what has previously been shown for PDH phosphorylation (71) and other protein phosphorylation sites (3).

Effect of acute changes in metabolic stress on Complex V activity in the pig heart in vivo and the perfused rabbit heart. Our hypothesis predicts that the activities of MOPCs are dependent on metabolic stress and therefore acutely track metabolic stress changes to maintain the metabolic homeostasis. To test this notion, we evaluated the impact of acute changes in workload on cardiac Complex V activity in biopsy samples. Because of material limitations, we opted to study Complex V activity since the above studies showed that it had the largest reserve capacity for generating ATP during increased workloads.

Heart and liver mitochondria reside in different cellular environments, which could alter MOPCs PTMs artificially during the isolation process. To minimize this effect, we obtained in vivo biopsy samples and analyzed them via BN-PAGE with minimal processing. Consistent with the isolated mitochondrial data presented in Fig. 2, the activity of Complexes IV and V in resting pig liver biopsies was increased by 1.9 ± 0.2-fold and 2.8 ± 0.1 (n = 4)-fold, respectively, relative to the heart (Fig. 5). Notably, the absolute reaction rates of Complexes IV and V were two to four times higher in isolated mitochondria than biopsy samples from the same animal (Fig. 5, C and D). This result suggests that the putative inhibitory PTMs associated with these MOPCs are labile, similar to what has previously been shown for PDH phosphorylation (71) and other protein phosphorylation sites (3).

Table 1. Comparison of mean porcine heart and liver contents using optical spectroscopy, DIGE and iTRAQ

<table>
<thead>
<tr>
<th>Mitochondrial Protein</th>
<th>Optical Spectroscopy</th>
<th>2D-DIGE</th>
<th>iTRAQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric acid cycle</td>
<td>ND</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td>Complex I</td>
<td>ND</td>
<td>2.2</td>
<td>3.7</td>
</tr>
<tr>
<td>Complex II</td>
<td>ND</td>
<td>1.5</td>
<td>1.4</td>
</tr>
<tr>
<td>Complex III</td>
<td>ND</td>
<td>4.2</td>
<td>4.8</td>
</tr>
<tr>
<td>Complex IV</td>
<td>4.5</td>
<td>3.9</td>
<td>4.5</td>
</tr>
<tr>
<td>Complex V</td>
<td>ND</td>
<td>3.4</td>
<td>3.8</td>
</tr>
</tbody>
</table>

The data from two-dimensional differential in-gel electrophoresis (2D-DIGE) and isobaric tags for relative and absolute quantitation (iTRAQ) represent the means of all subunits detected in the respective approach. Complete iTRAQ data is available in the online Supplemental Table 2. Ratios are expressed as heart/liver.

Table 2. Cyto a contents and estimated cytochrome rates at rest for liver and heart in allometric series

<table>
<thead>
<tr>
<th>Animal</th>
<th>Resting Heart MW, μmol O2·min⁻¹·g wet wt⁻¹</th>
<th>Resting Liver MW, μmol O2·min⁻¹·g wet wt⁻¹</th>
<th>Heart Cyto a Content (n), nmol/g wet wt</th>
<th>Liver Cyto a Content (n), nmol/g wet wt</th>
<th>Heart Cyto a Turnover, μmol O2·mmol cyto a⁻¹·min⁻¹</th>
<th>Liver Cyto a Turnover, μmol O2·mmol cyto a⁻¹·min⁻¹</th>
<th>Heart Relative Metabolic Stress</th>
<th>Liver Relative Metabolic Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>0.02</td>
<td>14</td>
<td>5.6°</td>
<td>35.2 ± 1.0 (3)</td>
<td>24.7 ± 3.0 (3)</td>
<td>0.4</td>
<td>0.22</td>
<td>1.2</td>
</tr>
<tr>
<td>Rabbit</td>
<td>2.5</td>
<td>4.5°</td>
<td>2.0°</td>
<td>33.2 ± 0.6 (4)</td>
<td>12.4 ± 1.6 (4)</td>
<td>0.14</td>
<td>0.15</td>
<td>0.4</td>
</tr>
<tr>
<td>Pig</td>
<td>50</td>
<td>2°</td>
<td>3.2°</td>
<td>31.5 ± 0.5 (5)</td>
<td>9.8 ± 0.7 (5)</td>
<td>0.07</td>
<td>0.12</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Cytochrome turnover×100/cytochrome content; References: °(47; 91), †(69), ‡(88). §(58). ¶(55).
average, dobutamine increased the rate-pressure product by a ratio of 2.4 \pm 0.6 compared with control, with a corresponding increase in Complex V activity of 1.8 \pm 0.3.

To generate a larger range of metabolic stress, we measured Complex V activity in the perfused rabbit heart during KCl arrest (minimal work), control, and dobutamine-stimulation conditions. Complex V increased proportionally with increasing metabolic stress in these hearts (Fig. 6, A–C). As observed for the in vivo pig heart studies detailed above, Complex V activity in the perfused rabbit heart increased by 2.4 \pm 0.6-fold with dobutamine stimulation, with an increase in heart rate of 3.4 \pm 0.5-fold. KCl arrest decreased Complex V activity to 0.5 \pm 0.1-fold of the control rate (Fig. 6, D–F). Thus these studies attained a dynamic range of in vitro Complex V activity (from KCl arrest to dobutamine stimulation) that exceed five-fold. It should be noted that the peak metabolic activity in saline-perfused hearts is likely restricted by oxygen delivery and limited afterload, which likely underestimates the peak cardiac workload. Nonetheless, these studies demonstrate that Complex V activity is acutely modulated by acute changes to cardiac metabolic stress and that these activity changes persist through BN-PAGE processing.

A role for posttranslational modifications in modulating Complex V activity. The current study revealed that per mole the baseline Complex V activity is approximately fourfold higher in the pig liver than the heart. This activity difference persisted throughout tissue processing and isolation of Complex V. The protein sequences for Complex V from the heart and liver vary only by an aspartate residue on the COOH-terminus of the \( \beta \)-subunit (56). Thus we reasoned that the source of the Complex V activity difference between tissues resulted from 1) different stoichiometry for the subunits of Complex V, 2) tissue-specific protein/metabolite interactions, or 3) covalent posttranslational modifications, including phosphorylation, oxidation, ADP-ribosylation, etc.

The subunit composition of MOPCs between the heart and liver was evaluated using iTRAQ and SDS-electrophoresis of Complexes I, IV, and V following extraction from BN-PAGE gels (an example for Complex V is shown in Fig. 7a). No significant differences in the subunit compositions were identified for any MOPC in the pig or mouse. iTRAQ was also used to screen for tissue-specific differences in small regulatory proteins across the MOPCs; no variations were identified.

Further attention was paid to Complex V and its inhibitory
protein IF1. Western blotting was used to assess the content of IF1, which was found to be slightly higher in the liver than heart (Fig. 7b), negating its role in augmenting Complex V activity in the liver. Importantly, for the interaction of a small regulatory protein to increase Complex V activity by approximately fourfold, it would have to approach a stoichiometric level with the Complex (68); such an abundant protein would be easily detectable with mass spectrometry or Western blotting techniques. From these analyses we do not believe that small protein interactions or differences in subunit stoichiometry can explain the increase in Complex V activity between the pig liver and heart.

We next focused on the role of covalent posttranslational modifications in regulating MOPC activity between tissues. Since previous studies have shown distinct 32P-labeling patterns for heart and liver mitochondria (4) and the current study revealed the largest activity difference for Complex V, we evaluated 32P-labeling in Complex V isolated from pig heart and liver mitochondria. As shown in Fig. 7C, most Complex V subunits were resolved by 2D SDS gel electrophoresis. In heart mitochondria, 32P-labeling was observed for the α-, β-, γ-, and d-chain subunits of Complex V (Fig. 7, D–G). By comparison, in the liver the γ-subunit was virtually dephosphorylated, whereas the d-chain was weakly phosphorylated. Most studies on the functional role of protein phosphorylation have found this modification to be inhibitory. Consistent with this notion, our findings suggest that the phosphorylation of several Complex V subunits in the heart may be responsible for its decreased activity relative to the liver. To directly correlate decreased activity in cardiac Complex V with phosphorylation status, we conducted a phosphatase screen. λ-Phosphatase was found to dephosphorylate the γ-subunit of Complex V in hearts, which resulted in more than a twofold increase in Complex V activity (Fig. 8, A and B). To ensure that the λ-phosphatase was not altering cardiac Complex V activity indirectly, we also compared the effect of λ-phosphatase on liver Complex V. Since the γ-subunit is inherently dephosphorylated in the liver, no increase in its Complex V activity was expected; this finding was confirmed (Fig. 8B). Importantly, these phosphatase studies also show that the difference in Complex V activity between the heart and liver are due to reversible covalent modifications and not merely irreversible damage that may have occurred during tissue processing.

Previous studies have also shown that heart mitochondria are dependent on Ca2+ for maximum activation (31, 61, 84). Utilizing this physiological condition, we correlated the 32P-labeling profiles of Complex V under control (0 μM free Ca2+) and activating Ca2+ levels (0.65 μM free Ca2+) with activity in the heart and liver. When intact heart mitochondria were incubated with Ca2+, the γ-subunit and the band containing the IF1, c, and g-subunits were dephosphorylated, which correlated with a 3.9 ± 0.5-fold increase in Complex V activity (Fig. 8, C and D). Similar to the phosphatase studies, Ca2+ incubation did not alter the phosphorylation status or activity of Complex V in the liver. It is important to point out that these Ca2+ effects were only observed when the Ca2+ incubation was
performed in intact heart mitochondria (i.e., no changes in \(^{32}\)P-labeling or activity were shown when isolated Complex V was incubated with Ca\(^{2+}\)). This finding suggests that Ca\(^{2+}\) is regulating processes, perhaps kinases and phosphatases, which reside in and require an intact mitochondrion.

**DISCUSSION**

In this study, proteomics, biochemical, and physiological approaches were combined to demonstrate that the molar activities of MOPCs scale with metabolic stress in the liver and...
heart for a given animal or across an allometric series. In addition, we demonstrate that the activity of Complex V is acutely modulated by cardiac workload and associated changes in metabolic stress. These data are consistent with the regulation of net MOPCs flux capacity by modulating protein content relative to the maximum energy conversion requirements of tissues (87). In tissues with low metabolic dynamic ranges such as the liver, MOPCs are operating at a relatively constant high metabolic stress and the changes in resting metabolic rate with body size are matched with mitochondrial mass consistent with previous studies (48, 69). In tissue with large dynamic ranges in energy conversion, such as the heart, the mitochondrial content is held relatively constant while the activity of the MOPCs is acutely modulated in proportion to the metabolic stress. In these dynamic tissues, we propose that PTMs provide a mechanism for reserving a significant fraction of MOPCs activity while the tissue is “rest.” During increases in energy conversion, alterations in PTM switch on the MOPCs to a more active form. This model of modulating MOPCs activity with work requirements parallels the previously demonstrated modulation of PDH activity delivering reducing equivalents to the cytochrome chain with work. The modulation of both the delivery of reducing equivalents and the MOPCs converting this energy into ATP provides a provides a theoretically balanced activation of energy conversion during work transitions.

Despite the large difference in resting metabolic rates between the pig and mouse heart, the current study found that the mitochondrial content is fixed at ~32 nmol cyto a/g wet wt across species. This translates to mitochondria composing ~21% of total tissue protein of the heart cell (assuming 140 mg of cellular protein/g wet wt (13) and ~1 nmol cyto a/mg mitochondrial protein; see RESULTS). These data appear to conflict with an earlier study by Hoppeler et al. (36), which concluded cardiac mitochondrial volume scaled with resting metabolic rate. However, replotted mitochondrial volumes by Hoppeler et al. as a function of heart mass (Fig. 9) results in a linear plot with a slope of 0.21 (0.22 if horse and cattle are included) consistent with our results. A similar analysis of Hoppeler’s data was made by Dobson et al. (25). These data demonstrate that the maximum aerobic capacity of the heart is nearly identical across species, which suggests that there is an optimal balance of mitochondrial and contractile elements in mammalian heart cells. A similar conclusion was recently reached in proteomic studies on the right and left ventricles, which showed that despite large differences in workload, the protein composition per myocyte was nearly identical between ventricles (66, 75).

In contrast to the fixed mitochondrial content of the heart, the mitochondrial content of the liver scales inversely with animal size. This finding agrees with the previous studies on the liver (48, 69). Since liver lacks a dynamic range for ATP production, its resting state does not require a large energetic reserve capacity or the need for a large dynamic regulation of MOPCs activity. Instead, these data imply that MOPCs and mitochondria content are elevated in the small animal liver to meet the increased energy conversion at a relatively fixed metabolic stress. We suggest that liver is able to increase its mitochondrial content since it does not have the spatial constraints of the cardiac contractile requirements.

The current study suggests that the maximum activity of Complex V is modulated directly by, or in concert with, the metabolic stress. We show that the total activity of Complex V matches the resting metabolic stress of the heart and liver across an allometric series as well as during acute changes in metabolic stress in the dynamic heart. Modulating Complex V maximum activity, either through protein content or PTM effects, allows the rate of ATP generation to be matched with ATP consumption in the metabolic homeostasis with essentially constant ADP and P_i, or ΔG_ATP. This is done by essentially modulating the effective maximum velocity of the reaction permitting an increase in flux with a constant substrate or thermodynamic driving force. Again, the maximum activity of Complex V can be modulated via protein content or the activity per mole. The content of Complex V must match the maximum energy conversion requirements to support this activity. In tissues with large energy conversion dynamic ranges, the molar activity of the Complex V seems to be additionally scaled to match the changing metabolic stress, presumably becoming fully active at the maximum energy conversion rates. Consistent with this later notion, we demonstrated that extracted Complex V activity was persistently inhibited in the resting heart relative to the liver but was able to be acutely activated in response to a dobutamine-induced increase in energy conversion requirements associated with a stimulation of inotropy and heart rate. Previous studies have demonstrated that this type of stimulation does not alter the ADP, P_i, or ΔG_ATP of the heart in vivo (42), consistent with the maintenance of the metabolic homeostasis. Based on these results, we speculate that kinetic regulation of Complex V activity is fundamental to the maintenance of the energetic homeostasis both chronically and
acutely. Specifically, we propose that PTMs provide a mechanism for altering ATP production at the level of the oxidative phosphorylation complexes of oxidative phosphorylation to match metabolic stress, without changing the conventional driving forces (i.e., $\Delta \Psi$ and $\Delta G_{ATP}$).

The fact that the changes in MOPCs activity persisted through the native gel isolation process is the best evidence that the activity of the enzyme was modulated by covalent PTMs or other strong associations. Persistence through isolation was also one of the earliest observations in the evaluation of PDH activity modulation by insulin action (22). While protein/metabolite associations or tissue-specific MOPCs subunit differences could also explain the persistence of MOPC activity differences, no such findings were identified in this study. Several PTMs have been shown to occur in matrix proteins, including the identification of phosphorylation sites for Complex V (1, 26, 51, 81) and Complex V (3, 4, 41). Our laboratory recently showed that the $^{32}$P-labeling patterns in pig heart and liver mitochondria are very different, independent of the protein concentration (4). Herein we focus on the differential phosphorylation of Complex V, which is persistent and reversible and therefore consistent with the criteria for a regulatory PTMs. Comparing tissues, $^{32}$P-labeling in the $\gamma$- and d-chain subunits is lower in the liver compared with the heart. Consistent with these events being inhibitory in the heart, we show that A-phosphatase and Ca$^{2+}$ treatments dephosphorylate the $\gamma$-subunit and activate Complex V. While the phosphorylation sites in MOPCs may be similar to bacterial autophostorylation process, our previous work on this topic did not reveal autophosphorylation for the $\gamma$-subunit (67), suggesting that it is regulated by a matrix kinase or phosphatase system. While further work is required to determine the actual phosphorylation site(s) for these Complex V subunits, their direct impact on activity, and the associated matrix kinases/phosphatase, the large activity differences revealed in this study provides a unique opportunity to differentially identify PTMs that dynamically influence MOPCs function.

Though skeletal muscle has an even larger metabolic dynamic range than the heart approaching 100-fold (34), we opted not to include this tissue in this study. We have found that the heterogeneity in the fiber type composition (16), remodeling by chronic exercise (35), and reliance on anaerobic ATP production in fast twitch fibers complicates the interpretation of biopsy data. In contrast, heart and liver are relatively homogenous in their composition even across species and highly dependent on mitochondrial oxidative phosphorylation. In addition, skeletal muscle exists as a continuum of different fiber types with different metabolic and functional capacities across species; thus, normalization across an allometric series would be difficult.

Using native gel electrophoresis, derived mitochondria complex activities is problematical in extrapolating to tissue mitochondrial function and discussion of this study limitation is warranted. Although most of the proteins believed to be associated with a given complex persist through the isolation process, the complex finds itself in a remarkably nonphysiological condition: the complex is outside of the membrane with variable amounts of associated lipid and without a $\sim$180 mV $\Delta \Psi$, which likely plays a major role in controlling fluxes through Complexes I, III, IV, and V. For example, isolated Complex I directly interacts with oxygen, which reacts with NADH to produce large amounts of ROS under these native gel conditions (15). Geometric relationships within the cristae and between complexes (see Zick, 2009 ZICK2009/id and Lenaz, 2007 LENAZ2007/id) that could dynamically modulate complex activity is eliminated and not evaluated by this approach. The substrate and metabolite milieu is also altered with some metabolites having very tight associations such as Mg-ADP in Complex V that has inhibitory effects (62). In addition, these in gel assays only sample partial reactions (Complexes I) or the reverse reaction (Complex V) in the absence of a $\Delta \Psi$. Thus these activities, which are extensively reported in the literature, really represent rough approximations of the in vivo enzyme activities. Another concern is that these assays are simply monitoring differential damage of the complexes during the isolation procedures. This is likely happening to some extent; however, we found that that MOPCs catalytic rate increased with processing of the samples, not decreased (see Fig. 5). Thus, if anything, the isolation is not damaging the complexes but removing inhibitory factors. Despite these drawbacks, reversible changes in enzyme activity, for example, in the pig heart in vivo (see Fig. 6), that persist throughout the BN- or CN-PAGE process imply that the complex has been rather significantly modified and likely reflects alteration to the enzyme activity under normal physiological conditions. This is particularly true for Complex V where the rotation of the $\gamma$-subunit is required for both synthetic and hydrolytic activity.

Another approach would be to isolate intact mitochondria from the different tissues and “probe” the different complex activities using different substrates and inhibitors (for example see Ref. 11). This is a common approach used by many in the field, including this laboratory. However, we have found that the mitochondrial isolation process (i.e., in situ perfused, ionic composition of initial isolation solutions, etc.) and the conditions selected for performing the experiments (i.e., carbon substrates and ion concentrations, including calcium, magnesium, sodium, etc.) all change the relative metabolic poise of this isolated organelle, making almost any result possible. This is particularly problematic for the carbon substrates used since each tissue is programmed differently for using different substrates (35). For example, Randle’s laboratory (43) first showed that the activity of PDH in mitochondria is much different immediately after isolation than after several minutes of rewarming in a “physiological” medium. We confirmed this phenomenon for many phosphorylation sites in the mitochondrial matrix by direct $^{32}$P-labeling; the largest fraction of $^{32}$P incorporation, including PDH, was found to occur during the rewarming period, which established a new, nonexchanging, steady state (4). These data imply that the phenomenon described by Randle et al. (71) for PDH is potentially occurring in many other PTM proteins. Indeed, we find a striking activation of complex activity in fully isolated mitochondria compared with rapid tissue biopsy samples (Fig. 5) or gross tissue homogenates. Additionally, we also have shown that Complex V activity in isolated mitochondria is sensitive to calcium. To observe a calcium activation for Complex V, calcium chelators (i.e., EGTA) are required in the initial tissue perfusion, and a calcium depletion step must be performed in the isolated mitochondria (84). The best way to maintain the matrix calcium at physiological levels in isolated mitochondria preparations has been a long-standing controversy, with no gold standard in vivo to standardize to. It should also be noted that
even an RCR of 10 is still much lower than the change in respiratory rate from an arrested heart to the maximum rate of respiration, which likely approaches 100. Thus the mitochondria are not as coupled in vitro as in the intact tissue. Taking these limitations into account, we decided that the direct isolation of MOPCs from tissues using BN-PAGE or CN-PAGE, which keeps the complexes intact and minimizes in vitro incubation conditions, was the best available methodology to evaluate our hypothesis.

Perspectives and Significance

In this study proteomics, biochemical, and physiological strategies were combined to evaluate the relationship between MOPCs content and activity in the liver and heart of an allometric series of animals and during work transitions in the heart. These studies reveal two different organ strategies in adapting to higher resting energy conversion rate with decreasing body weight. In the liver, with a very low energy metabolism dynamic range and associated high net metabolic stress, the MOPCs content scaled with the resting metabolic rate and maximum energy conversion rate. In the heart, the MOPCs content and apparent maximum energy conversion rate is relatively constant as a function of body size while the dynamic range of energy conversion decreases dramatically with decreasing body size. This later result suggests that the ratio of MOPC and contractile elements in the heart is nearly constant as a function of body size. This implies that this ratio is optimized with regard to the use of cellular volume to perform work and mitochondrial energy conversion rate in the mammalian heart. Though the heart MOPCs content was constant as a function of body size, we found that MOPCS activity was proportional to the resting metabolic stress, across the allometric series, as well as during step increases in work in the porcine and rabbit heart. The persistent nature of MOPCs activity changes in the isolated complexes is consistent with PTMs reversibly modulating MOPCS activity. We demonstrate that protein phosphorylation in Complex V may contribute to the acute regulation of this complex; however, the specific PTMs reversibly modulating MOPCs activity is unknown. PTMs in response changes in metabolic activity is important in maintaining the metabolic homeostasis of the heart, both as a function of body size as well as during the work transitions in larger animals. These studies suggest that the modulation of MOPC content and activity is critical for mammalian tissues to maintain their metabolic homeostasis as a function of body size as well as during dynamic changes in metabolic activity. The direct modulation of MOPCs activity by PTMs in response changes in metabolic stress is a novel hypothesis and may be extended to other tissues with large dynamic ranges in workload, including skeletal muscle, brain, and kidney. Whether compromises of the metabolic regulation of MOPCS activity with metabolic stress are important in different disease states of man is also unknown.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


