Modeling mitochondrial function

Robert S. Balaban
Laboratory of Cardiac Energetics, National Heart Lung and Blood Institute, Bethesda, Maryland

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Balaban, Robert S. Modeling mitochondrial function. Am J Physiol Cell Physiol 291: C1107–C1113, 2006.—The mitochondrial network represents a unique opportunity to apply mathematical modeling to a complex biological system. Understanding mitochondrial function and control is important since this organelle is critical in energy metabolism as well as playing key roles in biochemical synthesis, redox control/signaling, and apoptosis. A mathematical model, or hypothesis, provides several useful insights including a rigorous test of the consensus view of the operation of a biological process as well as providing methods of testing and creating new hypotheses. The advantages of the mitochondrial system for applying a mathematical model include the relative simplicity and understanding of the matrix reactions, the ability to study the mitochondria as a independent contained organelle, and, most importantly, one can dynamically measure many of the internal reaction intermediates, on line. The developing ability to internally monitor events within the metabolic network, rather than just the inflow and outflow, is extremely useful in creating critical bounds on complex mathematical models using the individual reaction mechanisms available. However, many serious problems remain in creating a working model of mitochondrial function including the incomplete definition of metabolic pathways, the uncertainty of using in vitro enzyme kinetics, as well as regulatory data in the intact system and the unknown chemical activities of relevant molecules in the matrix. Despite these formidable limitations, the advantages of the mitochondrial system make it one of the best defined mammalian metabolic networks that can be used as a model system for understanding the application and use of mathematical models to study biological systems.

oxidative phosphorylation; enzyme kinetics; nicotine adenine dinucleotide; fluorescence; ATP; nuclear magnetic resonance

AS WE BEGIN to put cells and organelles back together, after decades of dissecting the composition, structure, and function of their individual elements, it is clear that the complexity revealed by our experimental endeavors will expand to the point where it will be difficult to rely on intuition to generate hypotheses or even to interpret experimental data. This emerging acceptance of complexity in biology, often referred to as “systems biology,” has also rekindled interest in mathematical modeling as a tool. Several reviews and editorials have recently been constructed that outline the basic goals of this modeling activity (6, 9). Mathematical models are useful in keeping track of complex interactions in a network to aid the interpretation of data, to provide new hypotheses, as well as to suggest how to test a given hypothesis. Finally, the mathematical model is ultimately the final proof that the consensus impression of a metabolic network is correct by providing detailed predictions. That is, by taking all known kinetic and regulatory factors into account, a model can be constructed that accurately predicts the behavior of the network (88). An excellent example of using a consensus model was the study of yeast glycolysis that restricted the analysis to known kinetic parameters and substrate concentrations that was very effective in probing the discrepancies between in vitro kinetic data and in vivo behavior in this simple system (81). Many models have been created with too many parameters that are varied to fit macroscopic fluxes or limited experimental data. Those models rarely fail to fit a set of biological data and are quite prevalent in the literature. However, rarely does fitting a complex model provide much information on how well the current known set of pathways, reactions kinetics, and control mechanisms work, nor does it direct an investigator to an appropriate hypothesis to test or to measurements that need to be made. Running simulations with multiparameter models for finding limits and testing whether a complex hypothesis is theoretically feasible is a proven useful activity. However, I believe a better use of mathematical modeling is the model populated with the best current knowledge of a pathway and allowing the model to find the errors in this knowledge base to direct future efforts.

Many programs are currently underway to initiate quantitative models of cellular functions, and an incomplete list includes the following: the Virtual Cell (http://www.nrcam.uchc.edu/), the IUPS Physiome project (http://www.physiome.org/), the E-Cell Project (http://www.e-cell.org/), the Center for Modeling Integrated Metabolic Systems (http://www.csuohio.edu/mims/index.htm), the Silicon Cell (http://homepages.cwi.nl/~gollum/SiC/) and DiMSim (89). Though most large efforts have focused on the whole cell, the mitochondrion itself is uniquely suited to initiate this type of study on several levels. First, mitochondrion can function as autonomous process with a membrane barrier with measurable fluxes to provide simple limits for a mathematical model. Our knowledge of the molecular machines within mitochondria, although not complete, is extensive and expanding rapidly. This includes many of the enzymes of the citric acid cycle (17, 30, 46, 55, 56, 77, 88), oxidative phosphorylation (2, 25, 29, 39, 43, 57, 70, 84), and many other reaction sequences. Finally, and most importantly, measurements of the behavior of multiple constituents within the organelle can be monitored simultaneously with high temporal resolution to constrain a given model or to provide information about how to test a complex hypothesis generated by a model. In some circumstances it even permits the determination of enzyme kinetic data within the complex environment (19, 44). This latter ability to monitor numerous elements, dynamically, within the mitochondrial metabolic network is quite unique in most physiological or biochemical reaction sequences in mammalian systems. These properties of mitochondria have been appreciated by many

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investigators over the years who attempted to model one of the major functions, oxidative phosphorylation. Some of the earliest studies were designed to follow the stoichiometries of the reaction sequences as well as attempt to gain insight into control mechanisms (13–15), while more recent efforts have had similar goals but incorporated more detail about kinetic regulation and allosteric effectors (1, 4, 8, 26, 27, 49, 58). However, in general, most modeling efforts have simplified many of the reaction steps well beyond the detailed information that is available for given reaction steps, as discussed above, with simple rate equations containing some allosteric regulation with stoichiometric and thermodynamic limits. Though these are useful in establishing the feasibility of a particular mathematical construct to simulate a biological process, they have just begun to install the detailed molecular mechanisms for these reactions that are being currently generated. These simplified models are an important initial step in moving from working on individual proteins to ensembles of proteins with direct and indirect interactions which contribute to the function and regulation of mitochondria. Regrettably, simplification of mathematical models of complex processes is best done once the entire processes, or individual nodes, are well understood revealing the best opportunities for simplification to use in validation as well as ease of use. Many problems stand in the way of achieving the overall goal of an accurate model of mitochondrial function. The current barriers to generating this model, common to all, will be the focus of this review rather than a detailed, technical dissection of previous modeling efforts.

The mitochondrion may be much simpler than a whole cell; however, it is still a highly complex and interactive organelle that will likely require very sophisticated modeling tools to represent mathematically. Recent mitochondrial proteome studies have set the number of mitochondrial proteins on the order of 3,000 (60, 78, 85). To complicate this further, each tissue has a unique set of protein expression levels that will ultimately influence the overall function of the organelle. For example, Fig. 1 shows a two-dimensional (2D) gel dispersion of mitochondrial proteins from the heart and liver of a single pig where the cardiac proteins are labeled with a red fluorescent probe and the liver proteins are labeled with a green fluorescent probe. Since the same amount of protein was used from each tissue, similar protein level contents appear yellow, whereas proteins at higher levels in one of the tissues will reflect the color of that tissue. As seen in Fig. 1, the protein content of the liver and the heart are quite different, with very few proteins having similar expression levels. In general, the heart mitochondria are geared up for oxidative phosphorylation, while the liver has more protein associated with biochemical synthesis. A detailed description of these mitochondrial functional differences between tissues is found in Johnson et al. (43a, 43b) in this call for papers on the *Systems Biology of the Mitochondrion*. These data suggest that even tissue differences in protein expression will need to be taken into account in any qualitative description of metabolic pathways or quantitative understanding of mitochondrial function.

Even more confounding is the recent demonstration that the extent of posttranslational modification that modifies the activity of the matrix enzymes is extensive, especially with regard to protein phosphorylation (40, 72). It has been known for some time that protein phosphorylation is important in regulating mitochondrial enzyme activity, e.g., with the classic example of pyruvate dehydrogenase (38, 52). However, the extent of protein phosphorylation in the mitochondrial matrix has not been greatly appreciated. Figure 2 shows a 2D gel that is an overlay of $^{32}$P labeling and Coomassie blue staining of proteins from pig heart mitochondria that had been incubated with carbon substrates and radiolabeled inorganic phosphate ($^{32}$Pi) as the only source of extracellular phosphate. The $^{32}$P enters these energized mitochondria and is converted to ATP with $^{32}$P in the gamma position at very high (near 100%) specific activity, which permits the direct observation of matrix protein phosphorylation. Using radiolabeling or Pro-Q Diamond (Invitrogen) fluorescent dye approaches, several groups have been able to demonstrate an extensive phosphorylation of matrix proteins (40, 72, 74). Hopper et al. (40) were able to demonstrate extensive and dynamic protein phosphorylation in the mitochondrial matrix that was affected by calcium levels or by energization state. Many new protein phosphorylations have recently been described, although most of the specific sites remain unknown since $^{32}$P, and even Pro-Q Diamond labeling, is more sensitive in gels than mass spectroscopy is capable of determining sequences from extracted proteins (40).

In these studies of matrix protein phosphorylation all of the complexes of the cytochrome chain reveal dynamic protein phosphorylation sites; however, the functional significance of most of these events, as well as their associated kinases and phosphatases, remains essentially unknown. Indeed, the functional significance of protein phosphorylations throughout most of the metabolic networks of mitochondria, including the citric acid cycle, fatty acid, reactive oxygen species (ROS), and

![Fig. 1. Two-dimensional electrophoresis of mitochondrial proteins from the porcine heart and liver. The proteins were prepared as outlined by Hopper et al. (40) and differentially stained with Cy5 and Cy3 protein stains on lysine porcine heart and liver. The proteins were prepared as outlined by Hopper et al. (40) and differentially stained with Cy5 and Cy3 protein stains on lysine porcine heart and liver. The proteins were prepared as outlined by Hopper et al. (40) and differentially stained with Cy5 and Cy3 protein stains on lysine porcine heart and liver. The proteins were prepared as outlined by Hopper et al. (40) and differentially stained with Cy5 and Cy3 protein stains on lysine porcine heart and liver.](http://ajpcell.physiology.org/)

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amino acid metabolism, has not yet been fully explored. Even if only a fraction of the dynamic phosphorylations discovered have functional significance, modeling how these phosphorylations influence the net fluxes through this organelle will be a challenging but necessary task to understand the regulation of mitochondrial metabolism.

These proteomic approaches have essentially been providing valuable pieces of the jigsaw puzzle of mitochondrial metabolic pathways and functions. Many of the proteins identified in the mitochondrial matrix perform unknown functions (see Refs. 60, 78; and Johnson et al., Refs. 43a and 43b, in this call for papers), while we know of many important functions that we have not yet associated with a specific protein, such as the Ca\textsuperscript{2+} uniporter. Thus not all mitochondria metabolic pathways are fully delineated even as a chain of enzymatic activities. In many cases, metabolic activities can be predicted from the functional domains of a protein, rather than by assay, which could rapidly accelerate the functional identification. However, experimental confirmation of an enzyme’s activity will need to be performed with the structural analysis helping to decide which assay to perform. With this new information, a quantitative detailed model of the consensus networks would be extremely useful to help assemble this puzzle as new pieces, associated pathways, and regulatory aspects are identified.

Once the pathways have been established, the kinetic properties of the enzymes need to be applied to the model. For this task, classic proteomic analysis is not very helpful. To the credit of most investigators, they have attempted to fill in kinetic properties such as reaction mechanisms, substrate affinities, and allosteric interactions, rather than letting them float to help “fit” the model. However, most of the kinetic and allosteric information for mitochondrial enzymes are only available from purified enzymes. An excellent example of how allosteric and substrate effects can alter enzyme kinetics is the study of NADH generation by isocitrate dehydrogenase by Gabriel et al. (33), who demonstrated that NADH, NAD, ATP, ADP, and Ca\textsuperscript{2+} all influence the binding and interaction of the other substrates. Thus, for this enzyme alone, it would be difficult to model its ability to generate NADH without tracking all of these complex interactions. If similar data is generated for all the NADH generating enzymes of the citric acid cycle and fatty acid oxidation, then the requirements for a computation model for simple bookkeeping of the interactions of these effectors would almost be mandatory. Good starting points for modeling the citric acid cycle have been generated illustrating this complexity (26, 47). The next issue is to determine whether this in vitro kinetic data, as complicated as the interactions are, is really applicable to the matrix or membrane of mitochondria.

The issue of using in vitro enzymatic data for a model of the intact system is complicated by the fact that the in vitro reaction kinetics are rarely mimicked in the intact system (see examples, Refs. 11, 67, 81, 88). In our own laboratory, we attempted to extract and quantify all of the citric acid cycle enzymes in a single mitochondrial preparation (from porcine heart) using similar extraction procedures and uniform conditions. This approach was abandoned after we found that the extraction procedures affected the enzyme activities differentially, with many never reaching the activity for NADH generation that we know from mitochondrial oxygen consumption (S. French and R. S. Balaban, unpublished observations). In retrospect, this is not a surprising result based on the variability of mitochondrial enzyme activities in the literature. This discrepancy between in vitro and in vivo enzyme properties is likely due to many reasons, e.g., the extraordinary high ratio of...
substrates/products to enzymes in the matrix (3) and the remarkable low ratio used for in vitro studies. Furthermore, proteins within mitochondria are associated in complexes within and outside of membranes, as elegantly revealed using clear or blue native gel techniques (64). These enzyme complexes have been demonstrated to alter the kinetics of individual enzymes in the citric acid cycle and cytochromes chain (69, 71, 75), confounding the translation of in vitro enzymatic data for modeling purposes.

Another example is the initial oxidation of NADH in the cytochrome chain at complex I. In vitro, the apparent affinity of complex I for NADH is very low (on the order of 20 μM; Ref. 31), while numerous studies have shown that the affinity of NADH for oxidation in intact mitochondria is greater than millimolar (48, 59). Using NADH fluorescence lifetime measurements to estimate the binding of NADH in the mitochondrial matrix and in isolated membranes, the same order of magnitude shift in binding affinity was observed with intact mitochondria (>2 mM) and with isolated membranes (20 μM), matching the steady-state kinetics of oxidation (12). A similar discrepancy exists for inhibitory constants determined in vitro for NADH (on the order of 10–20 μM) that are very unrealistic for a matrix that rarely dips below 1.5 mM under in vivo conditions. The difference in NADH affinity at complex I is apparently not simply due to the presence or absence of a membrane potential and suggests a much more complicated interaction of NADH:NAD with this site. These examples suggest that relying on in vitro data for setting the kinetic parameters of individual enzymes is likely problematic. Thus kinetic measurements within the mitochondrial matrix or in intact physiological complexes are required. Thankfully, there are many measurements that can be made in the intact system that may help resolve some of these issues, as will be discussed below.

One of the unique aspects of studying mitochondria is the ability to simultaneously monitor numerous biochemical events as well as the matrix milieu with excellent time resolution. In addition to conventional rapid extraction biochemical assay approaches, one of the most powerful tools is optical spectroscopy and fluorescence as pioneered by Britton Chance (22, 24). Figure 3 shows a difference (anoxia vs. control) optical spectrum of a mitochondrial suspension in an integrating sphere to minimize scattering artifacts (16), which reveals the ability to, redundantly, monitor the redox state of all complexes involved in proton motive force generation for ATP production, with a time resolution on the order of milliseconds. The redundancy is the result of absorbance differences in the reduced and oxidized forms of many of the cytochromes in the 400–425 nm band as well as in the 500–620 nm band. Though this information is still limited with regard to the detailed mechanisms of ion ejection in oxidative phosphorylation, it is very useful for bounding what these complexes are doing in the intact system for modeling purposes. Other measures of the metabolic network are listed in Fig. 3 with references in the legend. Most of these approaches include the use of exogenously added optical probes for pH (SNARF, BCECF, etc.), Ca2+ (fura-2, indo-1, etc.), Mg2+ (mag-fura-2), ROS (Mitotracker red, Amplex red, etc.), membrane potential (ANNEPS, etc.), oxygen (hemoglobin, etc.) or using extra-mitochondrial electrodes including oxygen, NO, K+, Ca2+, Mg2+, pH, and various organic cations for determination of membrane potential. Other physical measures, such as light scattering for estimating volume and nuclear magnetic resonance (NMR) spectroscopy to follow several metabolites with 31P or 13C nuclides, fill out the current methodology. Many of these approaches have excellent temporal resolution, especially the optical approaches, and most can be arranged to make simultaneous measurements that are critical in the validation of mathematical models (see Refs. 48, 62, 80, 87). This latter aspect of these measurements is essential in evaluating kinetic parameters linking different aspects of mitochondrial function such as substrate oxidation, NADH and membrane potential.

**Fig. 3.** Monitoring of the metabolic mitochondrial metabolic network. The optical difference absorbance spectrum of control and hypoxic porcine heart mitochondria is adapted from Bose et al. (16). References to the other measurements include the following: original absorption spectroscopy of mitochondrial cytochromes (23), rapid optical monitoring of oxygen consumption (79), matrix pH (7, 28, 63, 76), membrane potential (61, 63), Mg2+ (45), Ca2+ (28, 37, 68), NADH and FADH fluorescence (19–21, 50), NADH turnover, ED-FRAP (44), carbon substrates 13C-NMR (51), ATP, ADP, and P, 31P-NMR (41, 42, 48), volume light scattering (35), ROS formation (53, 54, 73), and NO production (32).
generation, along with the ultimate formation of ATP. With the ability to measure these elements, the mitochondrial metabolic pathways are one of the best internally sampled networks available in the mammalian system and are a unique opportunity for modeling efforts.

Almost all of these measured parameters interact in very complex ways. Let’s consider complex I interaction with NADH again, which is a major crossroad of intermediary metabolism and oxidative phosphorylation. The redox state of NADH, potentially at its binding to active sites in the multi-enzyme complex I system, can be monitored via NADH fluorescence measurement using lifetime analysis (11). However, to understand how NADH levels are controlled, one needs to know the activity and kinetics of complex I, the proton motive force (which is a product of the complex I electrogenic reaction), the redox states of complexes II and III that accept reducing equivalents from complex I, the activities of dehydrogenases generating NADH, and, potentially, the phosphorylation of complex I and dehydrogenases via kinase phosphatase systems (40). This network rapidly expands to all factors that control these elements directly coupled to NADH production and utilization. These known regulatory factors include elements such as uncoupler proteins acting on membrane potential, calcium levels altering phosphatase/kinase systems, as well as all metabolites that alter the properties of enzyme systems as discussed earlier for isocitrate dehydrogenase. From this simple example, it is easy to see how modeling is almost mandatory to keep track of the interactions as well as to potentially predict the key measurements to make.

Even with the determination of these numerous elements in the network, there are many significant gaps in our measurement capabilities. The most significant are the chemical activities of most metabolites within the matrix and inner space. This is a major limitation, since in contrast to most ion determinations with optical probes and electrodes, we have not developed methods of detecting the chemical activity of metabolites; rather we can generally only measure total content. One approach for determining activity is to use near-equilibrium enzymes to reflect the relative concentration of metabolites (82, 86). However, no suitable enzymes for the NADH/NAD ratio seem to be available in the matrix (44, 66), nor for ATP/ADP and many other metabolites. Potentially, overexpressing reporter enzymes that could report chemical activities in the matrix, as the expression creatine kinase was used in the liver (5, 18) or luciferase for matrix Ca$^{2+}$ (68), might provide some useful insight. $^{13}$C magnetic resonance spectroscopy of metabolites in mitochondria might prove useful (51) since decreasing mobility increases the broadening of the $^{13}$C resonance and might provide good estimates of unrestricted metabolite pool sizes. Even useful may be the enhanced sensitivity of hyperpolarized $^{13}$C labeling (10, 36) in monitoring the in vivo tracer kinetics of individual metabolic or isotopic fluxes in the matrix. Naturally, the short magnetic lifetime of these hyperpolarized probes in the matrix environment may prove to be problematic when dealing with matrix metabolic rates on the orders of seconds.

The matrix and inner space phosphate and phosphate metabolites have also been the subject of extensive speculation, but very few measurements are available on chemical activity. The $^{31}$P-NMR has provided some insight in dense mitochondrial suspensions (41, 42, 65). However, the high viscosity of the matrix (~30% protein), together with paramagnetic metals that bind to most phosphate metabolites (42), makes the interpretation of matrix $^{31}$P-NMR line-width difficult with regard to chemical activity under in vivo conditions. One series of studies reported matrix P$_i$ values with $^{31}$P-NMR under specialized conditions in the perfused heart (34), but this approach has not been further investigated. As discussed above, genetically expressing enzymes, such as creatine kinase within the matrix where it is usually not expressed, could be used as a probe of metabolite chemical activity or even a perturbation if creatine can also be delivered in the matrix.

Another physical measure of metabolite mobility is fluorescence lifetime. NADH fluorescent lifetimes in the mitochondrial matrix have estimated that totally free NADH (~0.4 ns lifetime) is only ~20% in liver (83) and ~60% in heart (11), even though the concentration of NADH is relatively high (at ~3 mM). Though lifetime is an imperfect measure of chemical activity, these data are consistent with the notion that the activity coefficient of these metabolites is low, and using contents of NADH and NAD as surrogates for chemical activities is likely problematic.

The chemical activity of all metabolites and reactive species in the mitochondrial matrix is a fundamental measurement that must be addressed in the future to accurately describe the matrix milieu as well as to characterize the kinetics of various enzyme systems in the complexes likely formed in the matrix. The small volume and high protein/enzyme content of the matrix makes this compartment particularly susceptible to significant metabolite binding issues. The use of even the simplest computational methods, such as a dehydrogenase equilibrium assumption (for discussion, see Ref. 44), for determining activity coefficients is flawed in many cases. The problem is that multiple parameters affect net chemical activity including, transport, metabolism, and binding, which are poorly characterized. It is likely that determining matrix chemical activities directly would be more useful in bounding, if not understanding, the parameters of transport, metabolism, and binding rather than attempting to calculate activity from an incomplete model. Currently, the lack of direct information on the chemical activity of matrix metabolites is one of the major barriers in quantitatively describing the metabolic events in the mitochondrial matrix.

From this brief review, it is clear that we are far from having the information required to create an accurate model of the network associated with energy conversion in mitochondria. Excellent dynamic information is available on the complex redox states, membrane potentials, and ionic milieu of mitochondria. The major limitations are the activities of many of the matrix metabolites and the kinetics of the associated enzymes. However, it is time to begin this process and to incorporate the new information that is rapidly being generated by structural biology, proteomics, and genomics on this best model system of a mammalian biological reaction network. Without this modeling approach to guide the hypothesis-driven portion of this research as well as to prioritize efforts in measurement, it will be difficult to evaluate the consensus view on the operation of this relatively simple but still complex biological organelle.
REFERENCES


