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Domestication of the Cardiac Mitochondrion for Energy Conversion

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Abstract

The control of mitochondria energy conversion by cytosolic processes is reviewed. The nature of the cytosolic and mitochondrial potential energy homeostasis over wide ranges of energy utilization is reviewed and the consequences of this homeostasis in the control network are discussed. An analysis of the major candidate cytosolic signaling molecules ADP, Pi and Ca^{2+} are reviewed based on the magnitude and source of the cytosolic concentration changes as well as the potential targets of action within the mitochondrial energy conversion system. Based on this analysis, Ca^{2+} is the best candidate as a cytosolic signaling molecule for this process based on its ability to act as both a feed-forward and feed-back indicator of ATP hydrolysis and numerous targets within the matrix to provide a balanced activation of ATP production. These targets include numerous dehydrogenases and the F1-F0-ATPase. Pi is also a good candidate since it is an early signal of a mismatch between cytosolic ATP production and ATP synthesis in the presence of creatine kinase and has multiple targets within oxidative phosphorylation including NADH generation, electron flux in the cytochrome chain and a substrate for the F1-F0-ATPase. The mechanism of the coordinated activation of oxidative phosphorylation by these signaling molecules is discussed in light of the recent discoveries of extensive protein phosphorylation sites and other post-translational modifications. From this review it is clear that the control network associated with the maintenance of the cytosolic potential energy homeostasis is extremely complex with multiple pathways orchestrated to balance the sinks and sources in this system. New tools are needed to image and monitor metabolites within subcellular compartments to resolve many of these issues as well as the functional characterization of the numerous matrix post-translational events being discovered along with the enzymatic processes generating and removing these protein modifications.

Introduction

It is believed that the presence of the mitochondrion within eukaryotic cells is the result of a symbiotic relationship between early eukaryotic cells and some early forms of bacteria. The current role of the mitochondria evolved to include numerous novel processes in the cell such as specialized biochemical synthesis¹, lipid processing², reactive oxygen species signaling³, regulation of apoptosis⁴ and energy conversion via oxidative phosphorylation and substrate level phosphorylation.

The earliest studies of the relationship between cardiac work and mitochondrial ATP production, or respiration, demonstrated that mitochondrial ATP production is closely coupled to ATP hydrolysis at the myofibrils and active ion transport events associated with

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contraction. Since the extraction of oxygen is high and essentially constant, there is also a close coupling between tissue blood flow, mitochondrial respiration and work. These relationships are illustrated in Figure 1 from the study of Khouri et al 5 in the exercising awake dog. In these studies on canines working on a treadmill, a linear relationship between work, oxygen consumption and coronary blood flow was observed. Duncker and Bache 6 recently reviewed the relative contributions of heart rate (~60%), contractility (20%) and end-diastolic volume (Starling)(20%) to the increase in myocardial work linearly driving myocardial oxygen consumption. Though this phenomenon has been replicated over the years, the cellular mechanisms for these control processes remain unknown. In this review I will focus on acute regulatory mechanisms, the reader is referred to a recent review by Devin and Rigoulet on long term modifications of mitochondrial function⁷.

The initial models proposed that a simple feedback model of ATP metabolism products, that include ADP, Pi, and adenosine, would regulate the mitochondria via cytosolic ADP and Pi concentrations⁸⁻¹⁰, while adenosine would provide a feedback vasodilator to control flow¹¹. This was an extremely logical model since it provided the multifaceted control of respiration and blood flow via the breakdown of one molecule, ATP, intimately linked to the utilization of potential energy for cardiac work. However, in the late 1980's through 1990's non-invasive methods of monitoring high energy phosphate metabolites using nuclear magnetic resonance spectroscopy (NMRS) revealed that ATP and its hydrolysis products, ADP and Pi, did not change with large workloads when averaged over the heart wall¹²⁻²⁰. Evaluation of earlier classical biochemical "grind and find" experiments also confirmed this notion that changes in the concentration of ATP and its hydrolysis products were difficult to demonstrate with alterations in workload²¹⁻²³. Recent studies by Zhuo et al in a large work transition model in the pig confirms this notion²⁴. This "metabolic homeostasis" or maintenance of the free energy and concentration of key energy metabolites in the face of large perturbation of energy flux suggested that the simple feedback model of mitochondrial ATP production and blood flow may not be correct²⁵⁻²⁸. Possibly a more specific term for this process would be a potential energy homeostasis since it is generally being referred to the cytosolic potential energy associated with ATP. At the extremes of cardiac output in the anesthetized animal *in vivo*, with minimal venous return, alterations in the hydrolysis products of ATP, ADP and Pi, can be detected suggesting limits to the regulatory pathway approaching V_{max} .¹³⁻¹⁵ The extent of the potential energy homeostasis within the energy conversion metabolic pathway *in vivo*, and its limits, provided key information on the possible cellular control mechanisms for cardiac cellular respiration as well as coronary blood flow control.

The Cellular Potential Energy Homeostasis

As discussed above, ³¹P NMRS and chemical extraction studies demonstrated that in many systems, including man, that the ATP, ADP and Pi concentrations are very stable with variations in ATP utilization rates. This also concerned Hill shortly after the discovery of ATP challenging biochemist to demonstrate a breakdown of ATP during muscle contraction in a famous paper in 1950²⁹. Indeed, this challenge was essentially left unmet for a decade until multiple inhibitors of metabolism, iodoacetic acid, glycolytic inhibition, and fluoro-dinitrobenzene, creatine kinase inhibition, were employed by Cain and Davies³⁰ in 1962. This historical result together with the modern non-invasive techniques, demonstrate that the energy metabolism control network is poised to maintain ATP as constant as possible in the face of changes in ATP utilization.

What is the advantage of a potential energy homeostasis? The importance of phosphorylation potential in the function of the heart has been the subject of numerous articles in this journal³¹⁻³⁴. There are numerous advantages of maintaining the free energy

of ATP constant during changes in ATP turnover. First, and most obvious, is that the amount of energy available for work is determined by the free energy of ATP hydrolysis. If during increases in work the hydrolysis products of ATP are allowed to increase, then the free energy available for contractile work decreases when you need it most. This is demonstrated in Figure 2a where the effect of a 2% mismatch of ATP production with ATP utilization is plotted for the apparent ΔG_{ATP} and [ATP] in the canine heart, *in vivo*. As seen in Figure 2a, even small changes in ATP levels (~5% drop), and associated increases in hydrolysis products, will decrease the free energy available for contraction and ion transport by 5 to 6 kJ/mol. While a 20% change in ATP results in a drop of ~10 kJ/mole. In addition to decreasing the energy for contraction, the most sensitive energy dependent parameter may be the maintenance of the sarcolemma calcium gradient by the calcium pump (SERCA) as well as the Ca-Na exchange at the plasma membrane³⁵. This calcium gradient is critical for the initiation and power of cardiac muscle contractions. It has been appreciated for many years that one of the highest potential energy “stores” in the heart cell is in the calcium gradient³⁵, and any decrease in the free energy of ATP would impede SERCA from generating a Ca signaling gradient³⁶. This is also reflected in Figure 2b where the maximum sarcoplasmic reticulum calcium gradient is plotted along with the changes in ΔG_{ATP} . Thus, a small change in ATP content and ΔG_{ATP} results in a large decrease in the ability of the cell to accumulate Ca in the SR for signaling.

Secondary effects of having ATP and its hydrolysis products labile to changes in workloads would be reflected in the allosteric effects of these molecules on other reactions within the cell including numerous signaling processes associated with protein phosphorylation, redox state and synthetic reactions. If the ATP levels were not maintained all of these reactions would be modified by routine changes in cardiac workload, likely not a desirable condition.

Most of the measurements discussed to this point have been the gross NMR measures or grind and find determinations of ATP and its metabolites. What about the potential energy within the mitochondrion during work transitions? In most animals, the mitochondria volume is on the order of 25% of the cellular volume thus if large changes in ATP content in the mitochondria were occurring, one would suspect that this would be reflected in the gross ATP measurements and as discussed above no significant changes are observed. However, the ATP concentrations within the matrix could certainly be more labile than the total ATP content and be missed by the aforementioned volume and concentration differences as well as a broaden line width in ³¹P NMR measurements of metabolites in the viscous metal rich mitochondrial matrix.

More direct measures of the mitochondrial potential energy status can be obtained from optical measures of the NADH or FAD fluorescence *in vitro* or cytochrome c absorbance, *in vivo*. The most extensively studied system has been the mitochondrial NADH or FAD fluorescence signal. Recently we demonstrated that Complex 1 binding of NADH is one of the major sources of the NADH fluorescence lifetime enhancement in the heart mitochondrion³⁷ supporting the notion that using this signal is effective in determining the potential energy available for ATP production. Chance et al³⁸⁻⁴⁰ showed that the NADH redox state is highly dependent on the ADP and Pi levels, generally oxidizing with increases in ATP production rate driven by increases in ADP and Pi. Thus, the NADH redox state can be used as a monitor of the ADP and Pi levels at the mitochondrion in the intact cell. In several studies the mitochondrial NADH fluorescence signal or chemically determined NADH/NAD ratio in perfused hearts *in vitro* has been found to be essentially constant over normal work load transitions^{23, 41-43} suggesting that the potential energy homeostasis with workload extends into the mitochondrial matrix. Similar results have also been obtained by Brandes et al in isolated cardiac muscle⁴⁴ and by O'Rourke's lab in isolated myocytes⁴⁵. In contrast, Ashruf et al showed that a massive work jump from a vented non-working heart

to a fully working model transition that did result in a net oxidation of NADH 46. However, it is unclear whether even the cytosol phosphorylation potential was maintained under these conditions since others have shown that cardiac arrest dramatically increases the phosphorylation potential⁴⁷⁻⁴⁹ and increases mitochondrial NADH 50 by removing most of the work in a system that is normally never at rest.

No measure of the mitochondria NADH fluorescence has been determined *in vivo* in large animals during work transitions. Our own efforts failed in this regard due to the highly fluorescent elastin of the visceral pericardium interfering with the measurement⁵¹. However, optical measures of the cytochrome redox state have been made during work transitions in the canine heart. Arai et al 52 using optical reflectance spectroscopy determined that the redox state of cytochrome c remained constant during a tripling of the metabolic rate. These data taken together suggest that the potential energy homeostasis extends to the mitochondria matrix where most of the energy conversion processes of ATP production occur.

This extension of the potential energy homeostasis to the mitochondria matrix is important since it limits the types of models that may explain the regulation of mitochondrial ATP production. Extensive mathematical modeling has revealed that small micro-compartments of metabolites at diffusional barriers could exist that would result in a diffusion limited delivery of ADP and Pi without large changes in the bulk cytosol as detected with NMR or extraction methods (for example see 53). However the fact that the potential energy homeostasis extends to the mitochondrial matrix implies that ADP and Pi delivery alone is not being increased under this conditions. That is, if only local increases in ADP and Pi were increasing oxidative phosphorylation during work increases, then the classical oxidation of NADH and cytochrome c should be observed, no matter how small the metabolite pools are. Having said that, some experimental data consistent with metabolite compartmentation is compelling 54 and, like Ca^{2+} in the cytosol, the geometry of the heart cell may be generating metabolite gradients that play a role in this process. However, also like Ca^{2+} , the direct imaging of these metabolite gradients would greatly move this field forward as did the cytosolic $[\text{Ca}^{2+}]$ indicators^{55, 56}. New imaging modalities or metabolite sensitive probes may prove useful in this regard 57, 58. The simplest probe to develop may simply involve a fluorescence molecule sensitive to free phosphate for analysis of work transitions in the intact cell. Brune et al 59 has described a fluorescent protein capable sensitive to μM concentrations of Pi however the affinity is much too high for resting cytosolic levels. Potentially modifying this base protein may provide a novel tool in the investigation of compartmentation in the regulation of cellular energetics.

Another important component in this process is the creatine kinase reaction. It has been appreciated since the early experiments by Cain and Davies 30 discussed above that the creatine kinase reaction is essentially a limited buffer for ATP and ADP. Its limitation is linked to the fact that this reaction does not rely on a constant supply of energy but only redistributes energy from the dead end metabolites, creatine and creatine phosphate. Neither creatine or creatine phosphate has been demonstrated to have any allosteric or regulatory activity outside of being substrates for creatine kinase. Thus, the creatine kinase reaction is an effective temporal buffer of ATP contributing the potential energy homeostasis⁶⁰. Adding the creatine kinase reaction to the 2% imbalance between ATP production and synthesis presented in Figure 2, clearly reduces the short term impact of this imbalance in the calculated $^3\Delta G_{\text{ATP}}$ as well as ADP and ATP concentrations (Figure 3a). However, the actual changes in Pi are enhanced (See Figure 3b), suggesting that any short term imbalance between ATP production and breakdown will be reflected primarily in Pi and creatine concentrations over the short term and not ADP or ATP in the presence of a rapid creatine kinase reaction. Consequences of this relationship will be discussed further below. Another

near equilibrium enzyme distributed through the cytosol is adenylate kinase⁶¹ which removes 2 ADP molecules to reform ATP and AMP. In combination with creatine kinase and ATPase activity, this reaction generates another free Pi molecule by hydrolyzing ATP to AMP instead of just ADP, and limits the formation of ADP in the cytosol.

Creatine kinase (CPK) is located both at the myofibrils and mitochondria as different isoforms. Since the CPK equilibrium converts μM changes in ADP to mM changes in creatine the creatine could serve as a “shuttle”, or facilitated diffusion, of ADP contributing to the dissipation of any metabolite gradients. This property of CPK has been well appreciated in the cytosol or even within the mitochondria membranes coupled to other transport mechanisms^{62–67}. Indeed, a compromise of the CPK activity and associated facilitated diffusion has been implicated as a contributing factor in heart failure⁶⁸. However, it is quite surprising that the near total knockout of the CPK enzyme isoforms in mice results in modest if no measurable cardiac phenotype^{69–71}. The cardiac mitochondrial enzyme contents and total cellular ΔG_{ATP} was essentially unchanged in the knockouts as well as the ability to perform work transitions *in vitro* and *in vivo* where essentially unaffected. This is particularly surprising in the mouse where the same pressure volume work is being done as a large animal at 10 times the rate, placing the heart in the “resting state” near its theoretic V_{max} of ATP production²⁸. Thus, the strain on the ATP delivery system in the mouse at rest is equivalent to the ATP turnover of a sprinting dog⁵ and would predictably be highly susceptible to significant alterations in the ATP delivery system. The fact that even in skeletal muscle only burst performance is apparently affected in these CPK knockout mice along with aspects of Ca^{2+} handling and modest remodeling of the muscle structure might imply that the creatine kinase system is most critical in short burst activity, supporting the notion of a temporal buffer of the cytosolic potential energy. The impact on Ca^{2+} processing in these knockouts might reflect the high sensitivity to ΔG_{ATP} and rapid temporal responses required for Ca^{2+} sequestration discussed above.

There is another analogous finding related to a facilitated diffusion system for oxygen involving myoglobin⁷². Again in the highly active mouse where oxygen delivery is close to V_{max} conditions, the knockout of myoglobin has little or no effect on the normal function of the heart and is only associated with minor remodeling of the tissue^{73, 74}. Thus, these two facilitated diffusion systems, one for ADP and the other for oxygen seem not to be required for “normal” function or survival but may be more involved in transient peak performance issues that do not impact survival of a mouse living in a laboratory cage. This peak performance may have significant evolutionary importance since large amounts of both CPK and myoglobin protein is present in the heart and it is unlikely that these proteins are a biochemical appendix. The modest remodeling of the hearts in these situations do imply some impact on the normal functions that will need further investigation in these very interesting animals.

How is the Potential Energy Homeostasis Maintained in the Steady State?

As discussed earlier the major source of ATP in the muscle is oxidative phosphorylation and if the heart is able to vary the production of ATP at near constant levels of ADP and Pi then the simplest solution is simply to have the V_{max} of mitochondrial oxidative phosphorylation vary as a function of ATP hydrolysis. This would permit the generation of ATP at an appropriate rate without requirements for large changes in the reaction substrates ADP or Pi. Though this concept has been suggested for quite some time¹² the mechanisms associated with this balance must be quite complex since we now know that this homeostasis extends into the mitochondria matrix at the level of the NADH redox state, or primary driving force for ATP production, and the redox state of the cytochromes. This fact alone suggests that the control has to occur at multiple levels as suggested by numerous investigators over the years

using control theory or more mechanistic models 24· 75-81. To maintain the NADH/NAD ratio, cytochrome c redox state and the phosphorylation potential during large changes in electron flow in the cytochrome chain and generation of ATP at the F1-FoATPase would require a coordinated activation of multiple steps of oxidative phosphorylation within the matrix. In addition, this process has to occur rapidly (<1s) since even transient changes NADH 41 or high energy phosphates, including creatine phosphate, are not detected during work jumps 16. Despite the recognition that there are multiple sites of control within the oxidative phosphorylation reaction sequence, very little insight has been provide on the actual mechanisms associated with this distributed control. Currently, there is only one viable candidate for orchestrating this complex process, cytosolic free Ca^{2+} 82.

Role of cytoplasmic Ca^{2+} in regulating the potential energy homeostasis

It has been appreciated for years that the release of Ca^{2+} is critical for the initiation and control of cardiac contraction. By Ca^{2+} controlling contractile activity ATPase activity and being the primary substrate for the transport ATPase's of the SR and plasma membranes (via Na/Ca exchange and Na-K-ATPase) it is clear that cytoplasmic Ca^{2+} is an excellent marker of work related ATPase activity 83-85. Indeed the analysis of Duncker and Bache6 suggest that over 80% of the work associated with exercise (i.e. heart rate and contractility) is under cytosolic Ca^{2+} control. In addition, as shown in Figure 2b, any mismatch between ATP production and utilization would decrease ΔG_{ATP} . This would result in less Ca^{2+} uptake also increasing diastolic Ca^{2+} and decreasing the peak systolic Ca^{2+} level. The decrease in peak systolic Ca^{2+} provides a negative feedback signal for muscle contraction slowing ATP hydrolysis, while the increased average cytosolic $[\text{Ca}^{2+}]$ would provide a signal of extensive ATP hydrolytic activity to stimulate mitochondrial ATP production. Thus, the cytosol $[\text{Ca}^{2+}]$ is an appropriate cytosolic signal to reflect the extent of ATP hydrolysis in both a feedforward and feedback model. That is, whether cytosolic $[\text{Ca}^{2+}]$ is high from inducing contractile activity (feedforward) or in response to a decrease in ΔG_{ATP} due to excessive ATP hydrolysis (feedback) the mitochondria is receiving a positive signal to increase ATP production.

Randle provided a classical enzyme regulation model by demonstrating that pyruvate dehydrogenase(PDH) 86 is activated by a Ca^{2+} dependent dephosphorylation in the matrix. These studies were followed by the extensive studies by Denton and McCormack 87 and others 88 that clearly established that Ca^{2+} could activate several dehydrogenase activities in the intact heart through a variety of mechanisms. These dehydrogenases included PDH, α ketoglutaratedehydrogenase (KDH) and isocitrate dehydrogenase. This leaves only malate dehydrogenase and succinate dehydrogenase as the two sources of reducing equivalents not activated by Ca^{2+} . Panov et al 89 suggested that the dominate effect of Ca^{2+} on NADH generation in heart mitochondria is the allosteric activation of KDH which is consistent with data from our laboratory.

The Ca^{2+} dependent dehydrogenase activation by work has been demonstrated in several intact cell systems. Kobayashi and Neely demonstrated an increase in PDH activity with work in the intact perfused heart90. This activation of PDH demonstrates that the alterations in matrix Ca^{2+} were sufficient to activate PDH in this simple work transition experiment. Similar results have been obtained in human skeletal muscle with exercise91. These results suggest that the alterations in cytoplasmic Ca^{2+} and the mitochondrial Ca^{2+} transport mechanisms are sufficient for this enzyme activation. This is likely the result of the compartmentalization of the Ca^{2+} release sites of the SR with mitochondria as proposed and demonstrated by Rizzuto and Pozzan et al55· 56· 92. The activation of the dehydrogenases via Ca^{2+} has been demonstrated in the intact heart or heart cells to modulate the NADH redox state using a variety of methods 41· 44· 45· 83· 84· 93. Most notable is the disruption

of the potential energy homeostasis in the presence of mitochondria Ca^{2+} transport inhibitors such as ruthenium red^{94, 95}. Thus, it seems plausible given these extensive results that the supply of NADH can be matched to the demand for reducing equivalents for ATP production by using the same molecule, Ca^{2+} , that activates ATP hydrolysis to enhance mitochondrial NADH generation, in parallel.

How does the F1-F0 ATPase increase ATP production during work transitions when the substrates, ADP and P_i , as well as the driving force, reflected in the NADH redox state and cytochrome redox state, is constant? The simplest manner to accomplish this task would be to essentially increase the V_{max} , or decrease the effective “resistance”, of the F1-F0 ATPase permitting an increase in ATP production at a constant driving force.

Does Ca^{2+} modulate the F1-F0-ATPase? Das and Harris⁹⁶ found that Ca^{2+} activated the overall ATP hydrolytic activity in extracted F1-F0-ATPase from heart cells. This was reproduced in serial biopsy samples from dobutamine treated canine hearts, *in vivo*⁹⁷. Thus, changes in cytosolic Ca^{2+} apparently generated a post-translational modification of the F1-F0-ATPase that persisted as an alteration in ATP hydrolytic activity, *in vitro*. It is important to note that the addition of Ca^{2+} directly to isolated F1-F0-ATPase does not affect activity, thus the effect is more than a direct association of Ca^{2+} with the enzyme⁹⁸. Territo et al in a series of studies demonstrated that Ca^{2+} added to previously Ca^{2+} depleted cardiac mitochondria rapidly increased the velocity of ATP production by the F1-F0-ATPase at a given driving force (i.e. membrane potential)⁹⁹⁻¹⁰¹. Thus, Ca^{2+} is capable of increasing the capacity of ATP production by the F1-F0-ATPase at a constant driving force simultaneously with the increase delivery of reducing equivalents to the cytochrome chain via several Ca^{2+} sensitive dehydrogenases.

The dose dependence of the different actions of the Ca^{2+} on mitochondria and the SERCA pump was illustrated in experiments where the Ca^{2+} was added to mixtures of mitochondria and purified SR vesicles from the same heart⁸². Ca^{2+} was added to activate the dehydrogenases, F1-F0-ATPase and SERCA ATP hydrolytic activity to establish whether a potential energy homeostasis could be simulated in under these reconstituted conditions. Adding Ca^{2+} increased ATP turnover in this system, monitored as oxygen consumption, by activating SERCA. However, the increase in ATP generation was not associated with the classical decrease in NADH as electrons are withdrawn to support ATP production, the mitochondrial NADH was shown to be held constant or slightly increase (see Figure 4) with the addition of Ca^{2+} . The addition of Ca^{2+} in this system was contrasted with the graded addition of an ATPase (Apyrase) directly to the suspension generation ADP and P_i alone. Not only did NADH become more oxidized with increasing ATP turnover, but the overall maximum rate of ATP production was reduced due to the lack of activation of the dehydrogenases and F1-F0ATPase by Ca^{2+} . This rather simple reconstitution illustrates that extramitochondria Ca^{2+} driving the SERCA and activating oxidative phosphorylation can result in the required balanced activation to create the potential energy homeostasis during large increases in ATP production. In this reconstituted system a potential energy homeostasis was maintained over a 5 fold increase in ATP turnover.

Taken together these data imply that Ca^{2+} may be significantly contributing to the balancing of ATP production capacity during increases in ATP utilization, triggered by Ca^{2+} in the heart cell.

What is still yet to be resolved is the molecular mechanism associated with the activation of the F1-F0-ATPase by Ca^{2+} . Since this effect apparently persists in the purified protein extracted from mitochondria or cells activated with Ca^{2+} it is likely that some type of post-translational modification is responsible for this action.

Post-translational Modifications in Mitochondrial Energy Conversion Enzymes

One of the first examples of protein phosphorylation modifying enzymatic activity was the inhibitory phosphorylation of PDH, discussed earlier, that is modified by Ca^{2+} levels. Recently several screening studies have demonstrated that the dynamic phosphorylation of mitochondrial proteins may be much more extensive than previously appreciated 102-106.

Hopper et al 102 demonstrated that protein phosphorylation is extensive in the mitochondria matrix using a combination of protein phosphorylation dyes and ^{32}P labeling. Protein phosphorylation sites within all of the complexes of oxidative phosphorylation have been identified that may provide a mechanism of controlling multiple sites of oxidative phosphorylation using protein phosphorylation alone. Several of the subunits of the F1-F0-ATPase were found to be phosphorylated in the matrix. Extramitochondrial Ca^{2+} was found to dephosphorylate the gamma subunit along with PDH and MnSOD. Possibly this dephosphorylation of the gamma subunit may be partially responsible for the modulation of F1-F0-ATPase activity by Ca^{2+} . Others have also identified protein phosphorylation sites in the F1-F0-ATPase complex that could also contribute to activity modulation 106-107. The identification and functional significance of these protein phosphorylation sites in the F1-F0-ATPase are being actively studied in several laboratories around the world.

Another important issue with the growing evidence of an extensive protein phosphorylation network in the mitochondria matrix is the regulatory kinase/phosphatase system that is really quite poorly defined. Outside of a handful of kinases and phosphatases very few of these enzymes have been unequivocally located within the mitochondria matrix. The protein screens of the mitochondria reveal a very low number of kinases and phosphatases with uncertainty with regard to its location in the mitochondria or as contaminants from cytosolic structures 102-108-111. Thus, together with the analysis of the functional significance of these matrix protein phosphorylations, the associated kinases and phosphatases will likely be a significant subject of investigation over the next several years.

Another Ca^{2+} sensitive post-translational modification of the F1-F0-ATPase could be the association of regulatory peptides 112. The F1 inhibitory protein is one of these regulatory proteins 113 but its association with F1-F0-ATPase has not been linked to Ca^{2+} levels. Yamada et al 114-115 described a protein that inhibits F1-ATPase activity *in vitro* that is reversed by μM Ca^{2+} . This would provide a mechanism for Ca^{2+} to activate F1-ATPase activity by modulating this inhibitory protein activity. However, to my knowledge, no follow-up studies on this calcium sensitive inhibitory protein have been published. Boerries et al described a protein, S100A1, that has a Ca^{2+} dependent association with the F1-ATPase improving ATP production capacity 116. Interestingly, S100A1 over expression also facilitates the recovery from heart failure in model systems 117 possibly linked to this activity in the mitochondrion. The role of this protein in normal work transitions in the heart has not been investigated.

Over the last several years numerous other post-translational modifications, in addition to protein phosphorylation, have been identified in the energy conversion network of the mitochondrion using a variety of screening methods. These include acylation 118-119, palmitoylation 120, nitrosylation 121-123, oxidation 122-124, ADP ribosylation 125-126, and glycosylation 127. Many of these post-translational modifications occur in the complexes of oxidative phosphorylation most notable in Complex 1 and Complex V, however, the functional significance of these interactions is still under investigation. Many of these post-translational modifications have been evaluated under extreme conditions such as heart failure and diabetes, while very little work has been conducted on the normal work

transitions of the heart, where the role of most of these modifications is unknown. Clearly, there are many more post-translational modifications within the matrix energy conversion machinery than was imagined even a few years ago, with more likely to be discovered in the near future, increasing the potential complexity of the energy regulatory network. However, given the complex outcome of the maintenance of the potential energy throughout the cell, an extensive control network is likely necessary.

Feedback Role of ADP and Pi

Despite the fact that the concentrations of ADP and Pi remain essentially constant over normal workloads in the heart, at very high workloads^{13, 15} or different clinical conditions¹²⁸, the rate of ATP hydrolysis begins to exceed the ability of the control network to generate sufficient ATP without increasing the substrates ADP and Pi as well as reducing the ΔG_{ATP} to facilitate the rate of production. Another condition is when the potential energy generation capacity of the mitochondrion is externally influenced by the addition of substrates or hormones that induce a decrease in cytosolic ADP and Pi increasing the ΔG_{ATP} to “throttle” back the ATP production rate to match hydrolysis. This phenomenon has been observed with the addition of ketones *in vivo*¹²⁹ and with numerous substrates in the perfused heart^{14, 130-132}. These high work and substrate studies clearly show that ADP and Pi exert control on the ATP production rate in both directions as the classical feedback model suggested. What are the sites of activation of ATP production by ADP and Pi? Pi is transported into the mitochondria primarily in exchange for hydroxyl while ADP enters via the electrogenic adenylate translocase. Thus, both dissipate to some extent the overall proton-motive force with entry. Though ADP has been demonstrated to regulate numerous enzymes associated with NADH generation *in vitro*, Bose et al¹³³ found no evidence of increased NADH generation with the addition of ADP in the absence of Pi (Figure 5). Indeed a decrease in NADH is observed with ADP consistent with the reduction in residual matrix Pi by ADP phosphorylation (see below). Thus, the influence of ADP seems to be limited as a substrate for the F1-F0-ATPase. This is a somewhat surprisingly limited effect of ADP for what many believe is a key metabolite in the regulation of mitochondria function.

In contrast, the addition of Pi in the absence of ADP reveals a large increase in NADH generation and membrane potential (Figure 5A) as well as modifications to the redox equilibrium in the cytochrome chain limitations. The uncoupled respiratory rate is also dependent on Pi¹³³. The activation of electron transport by Pi is illustrated by the hyperpolarization of the mitochondrial membrane potential when oxidative phosphorylation is initiated by the addition of Pi after ADP (Figure 5B). Siess et al¹³⁴ noted a dependence of Krebs cycle function over physiological levels of phosphate in liver mitochondria with a cross-over point near succinate-CoA synthetase. Several other dehydrogenases have also been reported to be phosphate sensitive¹³³. Thus, it appears that Pi not only serves as a substrate for ATP synthesis but also stimulates the production of NADH in parallel resulting in an enhanced activation of ATP production capacity when compared to ADP alone.

The extensive nature of Pi's activation of oxidative phosphorylation is interesting when one considers which metabolites are likely to be changing during early mis-matches of ATP hydrolysis and production in the heart. As shown in Figure 3, in the presence of creatine kinase the changes in Pi are much earlier and larger than ADP in response to a mismatch of ATP production and utilization. Thus, the largest cytosolic error signal is Pi and not ADP. The regulatory network may be taking advantage of this fact by utilizing alterations in Pi to generate a balanced stimulation of oxidative phosphorylation, while the stimulatory sites available to ADP are limited. One issue with the notion of Pi playing a significant role in regulating oxidative phosphorylation is the apparent K_m for these processes in isolated

mitochondria (~0.5 mM) is generally lower than the resting Pi levels in most tissues¹³³. Thus, either the overall gain of the Pi process needs to be very high so that less than half of the activation curve is significant, or the kinetics of phosphate activation are much different in the intact cell. No information on the apparent affinity of oxidative phosphorylation for Pi is currently available in the intact cell.

A summary of the activation of ATP hydrolysis and oxidative phosphorylation by localized sarcoplasmic reticulum Ca²⁺ release is shown in Figure 6a. The only demonstrated Ca²⁺ activation sites are within the dehydrogenases and the F1-F0-ATPase. It is likely that other activation sites will be revealed in the future along with better molecular mechanisms associated with the system level activations demonstrated in the literature. I have not discussed the mitochondrial transport mechanisms since these were discussed in a previous review²⁷ in this journal. With a developing mis-match between ATP hydrolysis and synthesis, the earliest signal is predictably cytosolic Pi to supplement the effects of Ca²⁺ and to some extent ADP and creatine resulting from the creatine kinase reaction. The decrease in cytosolic ΔG_{ATP} will increase diastolic Ca²⁺ by inhibiting SERCA. The metabolic effects of Pi have been demonstrated to be even more distributed than Ca²⁺, activating NADH generation capacity, electron flux in the cytochrome chain, and a substrate for the F1-F0-ATPase as illustrated in Figure 6b. The specific interactions of Pi on these sites of oxidative phosphorylation are still under active investigation. These models suggest that both Pi and Ca²⁺ have the capacity to lead to an increase in energy conversion providing the potential energy required for ATP production under different physiological conditions. It is likely that this is still an oversimplification of this complex process that will be unraveled by future experimentation.

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Figure 1.

Effect of cardiac work on coronary blood flow and oxygen consumption in awake canines. Exercise was conducted on an 11° incline treadmill with rates approaching 16km/min. A) Represents the oxygen usage as a function of coronary flow over the exercise range. B) Represents the relationship between coronary oxygen utilization from using the left main or circumflex artery flow values and left ventricular work. C) Represents the oxygen extraction values as a function of running speed. All data adapted from Khouri et al 5.

Figure 2.

Simulations of the changes in the approximated ΔG_{ATP} with ATP hydrolysis and potential for SR calcium retention. During the arbitrary time points ATP breakdown was made to exceed production by 2%. The approximated ΔG_{ATP} (see 34) was determined using the following equation: $\Delta G_{ATP} = \Delta G_{ATP}^{\circ} + RT \ln([ADP][Pi]/[ATP])$. ΔG_{ATP}° was assumed to be 33kJ/mol. T was 310K. Resting metabolite levels were taken from literature for the canine heart 13, 135. A) Presents the ATP values in absolute values (M) solid line. Simulation is only shown for a 20% decrease in ATP. ΔG_{ATP} (kJ/M) is represented in a dashed line. B) A replot of ΔG_{ATP} (dashed line) with the calculated maximum ratio of $[Ca]_{cytosol}/[Ca]_{SR}$ (solid line) calculated from the following equation: $[Ca]_{cytosol}/[Ca]_{SR} = e^{(\Delta G_{ATP}/2RT)}$.

Figure 3.

Simulations as conducted in Figure 2 but with and without the addition of a creatine kinase equilibrium reaction. After each step in ATP hydrolysis (2% hydrolysis steps), the creatine kinase reaction was permitted to reach equilibrium through a numerical approximation. The creatine kinase equilibrium constant was assumed to be 1.66×10^9 . pH was 7.0. Other parameters were identical to those in Figure 2. A) Plot of ATP and the ΔG_{ATP} with, solid line, and without the creatine kinase reaction, dotted line. B) Plot of ADP and Pi with, solid line, and without the creatine kinase reaction, dotted line.

Figure 4.

Effect of Ca^{2+} (closed symbols) or Apyrase (open symbols) additions on the oxygen consumption and NADH/NAD ratio of an isolated porcine heart mitochondria and sarcoplasmic reticulum (SR) vesicle suspension. The Ca^{2+} was varied from a nominal value of 0 to 492nM free Ca^{2+} (calculated). The SR to mitochondria protein content was 0.5 in the suspension. Data adapted from 136.

Figure 5.

Effect of additions of Pi and ADP alone to State 4 porcine heart mitochondria. A) ADP added to State 4 mitochondria at the time indicated. The addition of Pi then initiated oxidative phosphorylation and associated oxygen consumption. B) Addition of Pi to State 4 mitochondria at the time indicated. Addition of ADP then initiated oxidative phosphorylation and associated oxygen consumption. Data is adapted from 133.

Figure 6a

Figure 6b

Figure 6. Schematic diagrams of the cellular energetic effects of Ca^{2+} and P_i in the heart cell. A) effect of sarcoplasmic reticulum (sr) release of Ca^{2+} on the ATP hydrolysis and oxidative phosphorylation. Activated sites are marked in red. Green lines indicate activation pathways. B) With a mismatch of ATP production with utilization P_i and creatine will increase. The

metabolic effects of Pi are highlighted in green with green lined indicating the path. Schemes are depicted on a electron microgram of a porcine heart cell fixed *in vivo*. Legend: TT: T-Tubules. DH: Dehydrogenases. C1: Complex 1.Q: Co-enzyme Q. C: Cytochrome c. C3: Complex 3. C4; Complex 4; C5: Complex 5 (F1-F0-ATPase). CPK: Creatine Kinase. ATPase: primarily myosin ATPase. s: SERCA Calcium pump. P_t;phosphate transporter. AT: Adenylate kinase. CrP: creatine phosphate. Cr: Creatine. Pi: inorganic free phosphate.