Regulation of Metabolism: the Work to Rest Transition in Skeletal Muscle

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ABSTRACT

The behavior of oxidative phosphorylation predicted by a model for the mechanism and kinetics of cytochrome c oxidase is compared to the experimentally observed behavior during the work to rest transition in skeletal muscle. For both experiment and model, when work stops the increase in creatine phosphate and decrease in creatine and inorganic phosphate concentrations ([CrP], [Cr], and [Pi]) begin immediately. The rate of change for each is maximal and then progressively slows as the increasing energy state ([ATP]/[ADP][Pi]) suppresses the rate of oxidative phosphorylation. The time courses can be reasonably fitted to single exponential curves with similar time constants. The energy state in the working and resting steady states at constant pO2 are dependent on the intramitochondrial [NAD\textsuperscript{+}]/[NADH], mitochondrial content, and size of the creatine pool ([CrP] plus [Cr]). The rate of change in [CrP] is linearly correlated with [CrP] and with [Pi] and [Cr]. The time constant for [CrP] increase, [CrP] in the resting and working steady states, and the rate of decrease in oxygen consumption are similarly dependent on the pO2 in the inspired gas (experimental) or tissue pO2 (model). Myoglobin strongly buffers intracellular pO2 below about 15 torr, truncating the low end of the oxygen distribution in the tissue and suppressing intra and inter-myocyte oxygen gradients. The predictions of the model are consistent with the experimental data throughout the work/rest transition, providing valuable insights into the regulation of cellular and tissue metabolism.

Key words: energy metabolism, oxidative phosphorylation, metabolic control, metabolic homeostasis, exercise, oxygen consumption, respiration
INTRODUCTION

Mitochondrial oxidative phosphorylation is programmed to set and maintain metabolic homeostasis (55-57, 62). As a result, oxidative phosphorylation provides robust, precise, and real time control of metabolism, responding to rapid changes in energy requirements as needed to return metabolism to the homeostatic set point. A precise metabolic set point combined with a robust system for maintaining that set point provides cross platform coordination of metabolism and allows cellular differentiation within an organism and development of complex structures. A model describing control of oxidative phosphorylation has been developed by combination of the rate expression for the mechanism of oxygen reduction by cytochrome c oxidase (42, 58, 59, 62, 63) with near equilibrium of the first two sites of oxidative phosphorylation (55-57). This model predicts behavior consistent with the steady state energy status and respiratory rates for a wide range of cells and tissues (55-57, 62). In order to gain insights into the dynamics of metabolic control and to more rigorously test the model, the predicted behavior is compared with that observed experimentally for physiologically important transients in energy metabolism. The larger the changes in metabolic rate, the more rigorously the predictive reliability of the model can be tested. To be considered successful, the behavior predicted by the model should not only be consistent with available experimental measurements but should also predict behavior that has not yet been measured but can be experimentally verified. The largest physiologically observed changes in metabolism, and those for which the most experimental data are available, occur when skeletal muscle begins or stops working. These involve increase or decrease in the rate of ATP utilization that can exceed 100 fold, providing a very demanding test for the model. The physiological changes in metabolic rate in other tissues are typically less than in muscle, but current evidence indicates the internal program of oxidative phosphorylation is the same for all tissues (55-57, 62). The differences among tissues are in the “tissue specific” parameters: content of mitochondria, size of the creatine phosphate pool, and dehydrogenases that provide the reducing equivalents.

In an earlier paper, the predictions of the model were shown to be consistent with experimental measurements of the metabolic changes that occur when resting muscles begin working (56). *A priori*, it might be expected that the rest to work and work to rest transitions would be mirror images, the same process but one the reverse of the other, as long as the work did not significantly fatigue the muscle. In this paper fatigue will be defined as “tiredness resulting from physical exertion” and assumed to be due to progressive changes not fully compensated over time. Moreover, since only metabolism is discussed, the work rate is assumed to be the rate of ATP utilization (in mM/sec), and not mechanical work. When muscle starts to do mechanical work the metabolic transition is driven by the increased rate of ATP utilization.
Metabolism responds to the decreasing energy state by increase ATP production until it again equals the rate of consumption and a new working steady state is achieved, but at a lower energy state. When muscle stops working, the rate of ATP consumption suddenly decreases relative to the rate of production and metabolism responds to the increasing energy state by decreasing the rate of production until it is again equal to the rate of production. Experimental measurements often show significantly different behavior for the rest to work and work to rest transitions. Although some of the reported differences can be attributed to muscle fatigue, differences have been observed even for short sub-maximal work efforts where fatigue is not a significant issue. When work is initiated, for example, oxygen consumption often shows a lag period (delay) during which the rate of increase starts slowly and goes through a period of accelerating rate before the maximum rate of increase is attained (4, 15-17, 22, 43, 44, 54). This contrasts with the work to rest transition where the decrease in oxygen consumption and increase in [CrP] begin immediately when work ceases. In the present paper, the behavior predicted by the model is compared to experimental measurements of the metabolic changes that occur when muscles stop working. The metabolic parameters that regulate the rate of mitochondrial oxidative phosphorylation (8-10, 21, 25, 40, 55-62) include: 1. the content of mitochondrial respiratory enzymes; 2. the energy state ([ATP]/[ADP][Pi]), with the creatine-creatine phosphate pool playing an important support role in muscle and brain; 3. the intramitochondrial [NAD⁺]/[NADH]; and 4. the intracellular pO₂. The present paper shows that the predictions of the model for oxidative phosphorylation are consistent with the available data for the work to rest transition. In the model, the contribution of each metabolic parameter in determining the rate and extent of the metabolic changes are readily quantified. As a result, predictions by the model provide insight into the quantitative relationships among the regulatory parameters that are very difficult to work out experimentally.

EXPERIMENTAL METHODS AND MATERIALS

The steady state rate expression for the mechanistic model for mitochondrial cytochrome c oxidase has been derived and shown to fit the kinetics of the reaction in isolated mitochondria (58, 59, 62, 63). The rate expression for the cytochrome c oxidase (site 3 of oxidative phosphorylation) has been extended by adding the first two sites:

\[ \text{NADH} + 2 \text{c}^{3+} + 2 \text{ADP} + 2 \text{Pi} = \text{NAD}^+ + \text{H}^+ + 2 \text{c}^{2+} + 2 \text{ATP} \]  

Equation 1 has been shown to be fully reversible and near equilibrium (8-11, 18, 21, 40, 55, 56, 57). Combining the equilibrium expression for equation 1 with the rate expression for cytochrome c oxidase results in a rate expression appropriate for oxidative phosphorylation:
\[
\text{NADH} + \frac{1}{2} \text{O}_2 + 3 \text{ADP} + 3 \text{Pi} \quad \Rightarrow \quad \text{NAD}^+ + H^+ + 3 \text{ATP} + \text{H}_2\text{O} \quad (2)
\]

where the NAD couple is that of the intramitochondrial space, the cofactor for the NAD linked dehydrogenases of the citric acid cycle, fatty acid oxidation, and amino acid catabolism. The derivation of the steady state rate expressions for cytochrome c oxidase and for oxidative phosphorylation as well as the programs for those rate expressions for MatLab (www.mathworks.com), can be found at ULAR: http://www.med.upenn.edu/biocbiop/faculty/wilson/index.html. The behavior predicted by the model has been calculated using MatLab and processed for comparison with the experimentally measured metabolite levels and graphed using Origin (www.originlab.com).

In order to compare the behavior predicted by the rate expression with experimental data for tissue metabolism, it is important to know and use the appropriate tissue specific parameters which include the mitochondrial content, ATP, ADP, Pi, creatine phosphate, creatine, and tissue pO2. Metabolite measurements from the literature have been used where possible, but in many cases missing parameters have been “filled in” using data from other papers. The "filled in" values most often needed were the mitochondrial content, total creatine concentration, concentration of inorganic phosphate [Pi], and pO2. It should be noted that the tissue specific parameters vary widely among muscle fiber types and where possible this should be taken into account. In the present paper, however, most of the data are from muscles with a mixture of fiber types and fit was to the data for the whole muscle. Although it would be interesting and informative to compare fiber types, this was considered outside of what could be discussed in this paper. The concentrations of ADP and AMP presented in this paper are for the free ADP and AMP, not total cellular concentrations, calculated assuming near equilibrium of the adenylate kinase and creatine phosphokinase reactions (13, 31). The P50 for oxygen binding to myoglobin in vivo is assumed to be 5 μM (3.6 torr). When the dependence on regulatory parameters other than pO2 was calculated, the pO2 in tissue was assumed to be 25 torr, a value considered representation of the mean value in the interstitrial space of normal muscle.

It should be kept in mind that mitochondria are particulate enzyme complexes and the internal components (cytochrome c, cytochrome a3, etc) are bound together with defined stoichiometry. When calculating the predicted metabolic behavior for tissues with different mitochondrial content, the calculations are made for 1 x 10^{-6} M cytochrome a (2 x 10^{-6} M cytochrome c), the conditions for which the model was fit to the data for suspensions of mitochondria. The calculated rates can then be scaled linearly with mitochondrial content i.e. doubling the mitochondrial content doubles the calculated rate. This allows the effect of mitochondrial content on tissue metabolism to be compared with the experimental measurements in tissues.
RESULTS

The effect of mitochondrial content on the changes in metabolism that occur when muscle stops working and metabolism transitions to a resting state. Figures 1A and B show the predicted changes in [CrP] when muscle working at a moderate rate stops working. The calculations were made for cytochrome c concentrations of 8 \( \mu \text{M} \) and 16 \( \mu \text{M} \) while the intramitochondrial \([\text{NAD}^+] / [\text{NADH}]\) ratio, total creatine concentration, and pO\(_2\) are held constant at 0.33, 46 mM, and 25 torr, respectively. In resting muscle, the basal rate of ATP utilization is assumed to be low, less than 0.01 mM ATP/sec (53), and in the working steady state this increased to 0.6 mM ATP/sec (Figure 1A) or 0.3 mM ATP/sec (Figure 1B). An ATP consumption rate of 0.6 mM/sec is approximately 6 \( \mu \text{mole O}_2/100\text{g tissue/min} \), a work rate about 60\% of the maximal oxygen consumption rate in human muscle (sedentary). The work to rest transition was initiated by stopping consumption of ATP. The [CrP] in the working steady state is higher for higher mitochondrial content. For a work rate of 0.6 mM ATP/sec the [CrP] levels for 8 and 16 \( \mu \text{M} \) cytochrome c are 15.9 mM and 20.8 mM, respectively, whereas for a work rate of 0.3 mM ATP/sec they are 21.6 mM and 24.6 mM, respectively. When work stops, increase in [CrP] begins immediately and then slows as the increasing energy state suppresses the rate of ATP synthesis by oxidative phosphorylation.

Experimental measurements of the increase in [CrP] with time after the muscles stop work are often reported as the time constant calculated by fit to a single exponential. The increase in [CrP] with time after work stops as predicted by the model are readily approximated by a single exponential (see Figure 1). The experimentally measured rate of respiration (rate of ATP synthesis) has been also reported to be linearly correlated with [CrP]. As shown in Figure 2, the rate of ATP synthesis (oxygen consumption) predicted by the model is linearly correlated with [CrP] over a wide range of rates, the slope of the straight line increasing in proportion to the increase in concentration of cytochrome c. Synthesis of creatine phosphate results in a stoichiometric decrease in inorganic phosphate ([Pi]) and creatine ([Cr]). As a result, decreases in [Cr] and [Pi] that occur also fit a single exponential with the same time constant as that for increase in [CrP]. Their concentrations, like [CrP], are linearly correlated with the rate of ATP synthesis. As an illustration, the [Pi] is plotted with the axis label on the top of the figure. The axis has been adjusted such that the points for [Pi] superimpose on those for [CrP].

Dependence of metabolism during the work to rest transition on the size of the creatine pool (creatine plus creatine phosphate). The creatine phosphokininase reaction and the [CrP]/[Cr] ratio are important to muscle function (28, 46). Figure 3 shows the effect of decreasing the creatine pool from 46 mM to 23 mM
while holding the other variables the same as for Figure 1, except that the intramitochondrial [NAD⁺]/[NADH] is 0.1 instead of 0.33. In each case, the increase in [CrP] and decrease in oxygen consumption begin as soon as the work stops. In the working steady state, the [Pi] is lower for the smaller creatine pool for the smaller creatine pool so that the [CrP]/[Cr] in the working steady state is lower. As for the 46 mM creatine pool, there is a linear correlation between [CrP], [Pi] and [Cr] and the rate of ATP synthesis over a wide range of rates.

**Dependence of the metabolic steady states and the rate of the changes that occur when work stops on the intramitochondrial [NAD⁺]/[NADH] ratio.** The intramitochondrial [NAD⁺]/[NADH] plays an important role in regulating oxidative phosphorylation (8-11, 21, 40, 55-57, 60). Figures 4A and 4B show the predicted dependence of the rate of ATP synthesis on [CrP] and [Pi] at intramitochondrial [NAD⁺]/[NADH] ratios of 0.033, 0.1, 0.33, 1, and 3. The pO₂, cytochrome c concentration, and creatine pools were 25 torr, 16 μM, and 46 mM, respectively. The [CrP] at each rate of ATP synthesis is higher as the intramitochondrial NAD pool becomes more reduced. An ATP synthesis rate of 0.3 mM/sec is achieved at [CrP] concentrations of about 28.5, 27, 25, 22.4, and 19.5 mM when the [NAD⁺]/[NADH] ratio is 0.033, 0.1, 0.33, 1, and 3, respectively. These ratios have been selected to encompass the ratios reported for muscles in vivo (14, 21, 40) as adjusted to the intramitochondrial pH of 7.5 (11). The values for the [CrP] are appropriate only for the a creatine pool of 46 mM, because the rate of ATP synthesis is dependent on the adenylate energy state, not [CrP]. The values of the [CrP]/[Cr][Pi] are 192, 142, 99, and 65 M⁻¹, respectively, equivalent to energy state ([ATP]/[ADP][Pi]) values of 2.7 x 10⁴, 2.0 x 10⁴, 1.4 x 10⁴, and 9.2 x 10³ M⁻¹. The dependence of the rate of ATP synthesis on the adenylate energy state is independent of the size of the creatine pool.

Figure 4B presents a 10x expansion of y axis of the curves in Figure 4A, showing that the linear correlation between the rate of ATP synthesis and [CrP] is observed only well above the resting rate and there is a pronounced nonlinearity as the resting steady state is approached. The rate of ATP consumption in skeletal muscle at rest is low, typically less than 1% of the maximal rate, or a cytochrome c turnover of about 0.5/sec. The combination of a quite reduced intramitochondrial NAD couple ([NAD⁺]/[NADH] near 0.1) and the low rate of ATP utilization results in the [CrP]/[Cr] ratio being high in the resting steady state.

**The pO₂ dependence on the metabolic changes that occur when muscle stops working.** The work to rest metabolic changes have been calculated for pO₂ values of 50, 25, 10, and 5 torr while holding the creatine pool, intramitochondrial [NAD⁺]/[NADH], cytochrome c concentration, and work rate at 23 mM, 0.2, 16 μM, and 0.6 mM ATP/sec, respectively (Figure 5). The initial [CrP] (in the working steady state) is lower for lower oxygen pressures. When work ends, [CrP] begins to increase with time. The initial rate of
increase is determined by the rate of ATP utilization/synthesis in the working steady state, and is not dependent on oxygen pressure. As [CrP] increases, the rate of ATP synthesis by oxidative phosphorylation progressively decreases and the increase in [CrP] slows until the resting steady state is attained. For pO2 values of 50, 25, 10 and 5 torr, the approximate [CrP] values in the working steady state (creatine pool of 23 mM) are 11, 10.4, 8.9, and 7.7 mM. The differences in rate of increase in [CrP] when work stops and in the level of [CrP] in the working steady state are expressions of the oxygen dependence of oxidative phosphorylation.

**The role of myoglobin in maintaining local oxygen pressure and sustaining oxidative phosphorylation.**

Human oxidative skeletal muscle contains 0.3-0.5 mM myoglobin (1, 64). This is sufficient to substantially increase the oxygen available for oxidative phosphorylation (expressed as nmoles/ml/torr) at oxygen pressures below about 30 torr. Figure 6 shows that at 30 torr the oxygen supplied by oxymyoglobin is approximately equal to that dissolved in the cellular medium. With continuing fall in pO2, the oxygen provided by oxymyoglobin (n mole/ml/torr) progressively increases, reaching 5, 10, 20, and 30 times that in the absence of myoglobin at pO2 values of 19, 11, 6, and 3 torr, respectively. The increase in oxygen that can be used per torr decrease in pO2 means that in working muscle the low oxygen part of the oxygen distribution remains significantly higher than it would in the absence of myoglobin. The presence of an oxygen buffer (myoglobin) decreases both local (intramyocyte) and regional (intermyocyte) differences in pO2. Buffering of pO2 by myoglobin suppresses the appearance of regions with oxygen pressures less the about 5 torr needed to maintain the energy state is critically important to muscle function, particularly at near maximal work rates.

**DISCUSSION**

The model is expected to be consistent with cellular energy metabolism throughout the regulatory range up to near, but not including, the maximal rate. From an evolutionary standpoint, the maximal rate is the design limit, presumably because the "cost" of further increase was greater than the evolutionary advantage. As the maximal rate is approached, the rate becomes dependent on limitations in oxygen delivery and other factors not directly involved in regulation of oxidative phosphorylation. These are not included in the model, so the model is not able to predict behavior near the maximal rate. For this reason, in this paper the presentation is limited to metabolic rates of up to 60-80% of the maximal rates. The dynamic range covered by the model is large, from rest to rates 80 times the resting rate. This encompasses most experimental exercise protocols, except those designed to determine the maximal rates for individual muscles. Similarly, the model does not
predict any of the progressive changes observed with sustained or very hard work, such as glycogen depletion or lactate accumulation.

**Comparison of experimental data with the behavior predicted by the model:** When skeletal muscle stops working, the time dependence of the resulting increase in [CrP], both experimentally observed (see Table 1) and predicted by the model (Table 2), can be fitted reasonably well by a single exponential. Table 1 shows the reported time constants for the increase in [CrP] with time after work stops while Table 2 shows the time constants predicted by the model for cytochrome c contents of 8 and 16 μM and [NAD⁺]/[NADH] ratio of 0.33 and a pO₂ of 25 torr. The predicted increases are not single exponentials because the value of the time constant obtained by fit to a single exponential increases with decreasing working steady state. The deviation from a single exponential is, however, small enough that the noise in experimental data is sufficient to obscure them. The times constants calculated for the experimental data and for the model are in agreement within the uncertainty in the measurements of mitochondrial content and other tissue specific metabolic parameters. Decrease in the rate of oxygen consumption is linearly correlated with the increase in [CrP] for both experiment (3, 12, 19, 26, 34, 36, 37, 39, 48, 50, 51) and model. It should be noted that fit of the increase in [CrP] with time by a single exponential indicates there is a linear correlation between [CrP] and rate of ATP synthesis (and rate of respiration). The creatine kinase reaction is not, however, involved in the regulation of oxidative phosphorylation except through equilibration with the adenine nucleotide pool. The model predicts that the [CrP] in the working steady state at constant pO₂, work rate, and intramitochondrial [NAD⁺]/[NADH] increases with increasing mitochondrial content. This is consistent with the experimental observations in different muscle types (5, 29) and with endurance training of muscles (6, 7, 27, 35). The rate of oxidative phosphorylation (rate of ATP synthesis and rate of respiration) increases with decreasing energy state. The rate of ATP production for any specific metabolic condition is proportional to the mitochondrial content, so increasing the mitochondrial content by 2 fold decreases the required cytochrome c turnover number by half. The transition from working steady state to the resting steady state occurs more quickly with increasing mitochondrial content, consistent with experimental observations in different fiber types and when comparing sedentary and trained animals (6, 7, 35, 38). In rats, the cytochrome c content of skeletal muscle is approximately 12 nmole/g wet weight (7, 49) and in human muscle it is reported to be about 5.8 nmole/g wet weight in upper leg muscle (2) or approximately 70% of that in rat skeletal muscle (65). This suggests that in sedentary humans the cytochrome c level is near 8 μM with 16 μM being more appropriate for trained individuals. Humans have a wider range of muscle oxygen consumption rates than rats, and when comparing sedentary individuals to in elite athletes the differences in mitochondrial content might be expected to be greater than for sedentary verses trained rats. If
the mitochondrial content is included when calculating the predictions for the model, they are consistent with the available experimental data.

**The dependence of muscle metabolism on local oxygen pressure:** Measurements of the rate of oxygen consumption by cells typically show that the rate is maintained until the pO$_2$ falls below about 5 torr, with the rate decreasing to 50% below 1 torr (32, 45). The model predicts that if the energy state and reduction of the intramitochondrial NAD couple are held constant, the respiratory rate begins to decrease at a pO$_2$ above 35 torr and to decrease to 50% of maximal rate by about 15 torr. In vivo, however, the energy state and intramitochondrial [NAD$^+$/[NADH] are regulatory variables. The function of oxidative phosphorylation is to synthesize ATP at the rate that it is being consumed, and the reactions that consume ATP (ion transport, mechanical work, molecular synthesis, etc) are generally not very, if at all, dependent on the oxygen pressure. As pO$_2$ decreases, the respiratory rate begins to fall only when the decrease in energy state and intramitochondrial [NAD$^+$/[NADH] no longer sustain the rate of ATP synthesis. The changes in energy state and [NAD$^+$/[NADH] needed to decrease the P50 for oxygen from 15 torr to less than 1 torr can be calculated. Figure 7 shows the increase in reduction of the intramitochondrial NAD couple, as a percent as NADH, predicted to hold the respiratory rate constant as the pO$_2$ decreases from 35 torr to < 5 torr when the energy state is constant. For a cytochrome c turnover number of 4/sec, the increase in NADH is from about 70% to 95%, an increase of 25%. This increase is sufficient to keep the rate of ATP synthesis constant as the pO$_2$ decreases from 50 torr down to 5 torr (decreasing the P50 to < 1 torr). The dependence is on the [NAD$^+$/[NADH], not [NADH], so as the reduction of the NAD couple at 50 torr increases above 50% the increase in NADH needed to hold the rate constant is smaller. For a cytochrome c turnover of 8/sec, for example, the increase is from 84% to 94%.

Decrease in energy state can also maintain the rate of ATP synthesis as pO$_2$ decreases. The decrease in [CrP], at constant intramitochondrial [NAD$^+$/[NADH], that would hold the respiratory rate constant from 50 torr down to 5 torr is shown in Figure 8. The calculations are for a creatine pool of 46 mM, an [NAD$^+$/[NADH] of 0.2, and cytochrome c TNs of 3, 5, and 10/sec. The [CrP] in the working steady states at a pO$_2$ of 50 torr are 30.5, 29.8, and 28.5 mM, respectively and as the pO$_2$ decreases to 5 torr, [CrP] decreases to 28, 26.9, and 24 mM, respectively. The decreases in [CrP] are 8, 9.7, and 16%, respectively. The oxygen dependence of oxidative phosphorylation predicted by the model is consistent with the P50 for oxygen measured in cells and tissues. Isolated mitochondria are damaged during isolation and this introduces an energy dependent “leak” reaction that confounds both experimental design and data interpretation. In vivo both the intramitochondrial [NAD$^+$/[NADH] and the energy state would compensate
for the decrease in pO2 in order to hold the rate of ATP synthesis equal to the rate of consumption, and the observed changes in each would be smaller.

**The role of myoglobin in muscle metabolism:** It is clear that myoglobin plays an important positive role(s) in muscle function or it would not be so widely conserved in evolution. Myoglobin is not present in tissues such as kidney, brain, and liver although these tissues have high metabolic rates and their content of mitochondria is similar to that of skeletal muscle. Thus, advantages proposed for myoglobin need to be specific for muscle. Oxygen buffering by myoglobin is well matched to the oxygen requirements of oxidative phosphorylation. Half saturation of oxygen binding by myoglobin results in strong buffering of the pO2 is in the critical range for oxidative phosphorylation, where further fall in pO2 compromises energy metabolism. The presence of myoglobin is much more important to muscle than other tissues because of the rapid changes in rate of oxygen consumption (ATP utilization), the high rates reached, and the compression that occurs during strong muscular contractions, especially sustained tetanic contractions, which can suppress blood flow and result in transient periods of ischemia.

One additional function for myoglobin is to store oxygen. The myoglobin content of muscle in diving mammals, such as the sperm whale, is approximately 10 times higher than in human muscle (1, 41). In the absence of myoglobin and at a pO2 of 20 torr, the dissolved oxygen is just 28 nmole/ml, equivalent to 0.168 mM ATP. In diving mammals, with approximately 5 mM myoglobin, oxymyoglobin in muscle provides a store of oxygen that is 200 times that of the dissolved oxygen (equivalent to 30 mM ATP). This is a substantial increase, particularly when account is taken of the large volume of muscle, only part of which is used for diving. The oxygen from muscles that are less active during the dive reenters the blood and is transported to the working muscles. This is consistent with oxygen bound to myoglobin playing an important role in making possible the long dive times use for feeding (41). This increase in myoglobin content is an evolutionary adaptation in which the levels for myoglobin in muscle increased in response to a specific environmental challenge.

It has been suggested that myoglobin is present in order to facilitate oxygen diffusion (64). This view assumes that oxygen delivery to the mitochondria in muscle is diffusion limited and that diffusion of myoglobin provides a parallel pathway for oxygen to reach the mitochondria. Theoretical calculations indicate that myoglobin diffusion may increase the oxygen flux by as much as a factor of 4 (64). If delivery of oxygen to the mitochondria were diffusion limited, however, this would eliminate the role of myoglobin as an oxygen buffer, replacing a large benefit of myoglobin with a much smaller one. Diffusion limitation of the delivery of oxygen to the mitochondria may be a significant factor in determining the maximal work rate of muscle, but is not a factor under normal working conditions.
**Summarizing for the role of myoglobin:** The oxidative skeletal muscle of land animals has 0.3 to 0.5 mM myoglobin. This is sufficient for it to act as an oxygen buffer that; A) truncates the low end of the oxygen distribution in working muscle in order to keep the local pO$_2$ values from falling into the region where the function of oxidative phosphorylation is severely compromised, and B) flattens distribution of oxygen within and among myocytes (52), helping to coordinate their energy states for optimal work efficiency. It is also a store of oxygen that helps to delay failure of muscles during the ischemic episodes induced by tetanic contractions. These functions of myoglobin are important for optimal performance of oxidative red muscle, and myoglobin content of muscle correlates with mitochondrial content, high in oxidative red muscle and very low in glycolytic white muscle. These functions are not needed for other tissues, providing the evolutionary bases for the presence of myoglobin in muscles.

**The role of glycogen and lactate production in muscle:** Glycogen is capable of providing substantial amounts of ATP in addition to that formed by oxidative phosphorylation, 1.5 ATP per lactate produced. If the concentration of lactate in the muscle tissue becomes too high, however, the resulting acidosis can limit muscle function and, in extreme cases, cause tissue damage. When the pO$_2$ is above about 5 torr and the work rate is not too high, i.e. oxidative phosphorylation can maintain the [CrP]/[Cr] above about 0.5, glycogenolysis is regulated to provide sufficient pyruvate for oxidative phosphorylation (the citric acid cycle) plus a small excess that appears as lactate. In normoxia this lactate production is small, but decreases if oxygen in the inspired gas is increased to 100% and increases if the pO$_2$ in the inspired gas is decreased (20, 24, 30). As work rates increase to maximum and/or pO$_2$ decreases to below about 5 torr, the [CrP]/[Cr] falls progressively. Lactate production from glycogen increases to compensate for the decreasing ability of oxidative phosphorylation to produce ATP at the needed rate and energy state. These lower energy states are accompanied by a large increase in [AMP], an activator of both glycogenolysis and glycolysis, and this likely plays a substantial role in increasing lactate production. Under moderate exercise conditions with sufficient oxygen delivery, glycogen provides pyruvate for sustaining oxidative phosphorylation and production of lactate is low. Under conditions of maximal work or deficiency in oxygen delivery, lactate production from glycogen provides an additional source of ATP to help sustain muscle function. The large increase in lactate production occurs when the energy state decreases to less than $10^3$ M$^{-1}$. This is only 1% of the resting value and yet the muscle contractions remain strong. The reported efficiency of muscle fiber conversion of the energy available in ATP to mechanical work is less than about 60% (60). Decrease in energy state by $10^2$ would lower the free energy available in ATP hydrolysis by only about 20% so sufficient energy is available in ATP hydrolysis to support maximal force generation.
The evolutionary impact of oxidative phosphorylation and mitochondria. An important feature of all plants and animals with extensive cellular differentiation is that their cells contain mitochondria and rely on oxidative phosphorylation as their primary metabolic energy source. This implies that higher plants and animals are derived from a common organism formed when a single celled bacterium that had developed oxidative phosphorylation was incorporated into a host organism (a eukaryote?) (33). With evolution, real time control of metabolism was taken over by the program intrinsic to oxidative phosphorylation while genetic control remained with the host. Mitochondrial oxidative phosphorylation provided both precise control of metabolism and high efficiency in converting the energy available the oxidation of metabolites into ATP. The sensitivity and precision of the control is extraordinary. As previously emphasized, oxidative phosphorylation holds the average value of the energy state nearly constant and within a narrow range despite the fact that this value more than 10^{10} greater than the equilibrium value (55-57). The sensitivity and range of control are also extraordinary. In muscle with a constant [NAD^+]/[NADH], for example, the rate of ATP synthesis increases about 20 fold when [CrP] decreases only 24%, and the increases in [ADP] and [Pi] are only 90% and 150%, respectively. Robust cross platform control of metabolism allowed individual cells to differentiate and form groups (tissues) with specific functions and that had to work together for the benefit of the whole. It is reasonable to suggest that successful integration of the oxidative phosphorylation into metabolism contributed significantly to the "Cambrian explosion", a relatively short period of time about 540 million years ago during which most of the current animal phyla developed. Importantly, mitochondrial oxidative phosphorylation is associated with the formation of specialized tissues in both animals and plants. The timing of the appearance of differentiation in plants is not as well documented as for animals, but may have occurred later. In plants, differentiation may not have proceeded until chloroplasts were formed and photosynthesis could be controlled separately, but in coordination with, oxidative phosphorylation.

In summary: In experiments, where both the energy state and [NAD^+]/[NADH] are expected to change, the increase in intramitochondrial [NADH] and decrease in [CrP] are difficult to measure accurately even under ideal conditions. It has not yet been possible to measure all of the regulatory parameters for oxidative phosphorylation, pO_2, energy state (either [ATP], [ADP], and [Pi] or [CrP], [Cr], and [Pi]), intramitochondrial [NAD^+]/[NADH], cytochrome content, and respiratory rate in the same experiment. The model developed earlier (55, 56, 59, 62, 63) has been shown to be consistent with the wide range of available experimental data, including the rapid and large metabolic transitions from rest to work (56) and work to rest (this paper). As such, the model is a robust mathematical representation of the regulation of oxidative phosphorylation, and therefore of energy metabolism in eukaryotic cells. This opens a window through which to “see” how the individual parameters work together to form an integrated and precise control
system. The model therefore provides important insight into how metabolic homeostasis is set and maintained. The precision with which the program in oxidative phosphorylation sets the metabolic energy state in eukaryotes is remarkable, as is its ability to maintain homeostasis while allowing for large swings in metabolic rate. It is easy to see how this precise, robust, across platform control of metabolism could provide the basis for cellular differentiation and the development of complex organisms.

REFERENCES


**FIGURE LEGENDS**

**Figures 1A, B. The changes in metabolism when muscle stops working as calculated for different concentrations of cytochrome c (mitochondrial content).** The calculations were made for a total creatine (creatine plus creatine phosphate) concentration of 46 mM, and intramitochondrial [NAD⁺]/[NADH] of 0.33, and a pO₂ of 25 torr. The behavior for cytochrome c concentrations of 8 and 16 μM are presented for muscle working at 0.6 mM ATP/sec (Fig 1A) and 0.3 mM ATP/sec (Fig 1B). The levels of [CrP] in the working steady state increase with higher content of mitochondria and with decrease in work rate. The [CrP] begins to rise immediately when work stops. The rate of increase slows due to the increasing energy state with an
asymptotic approach to the resting steady state levels. The increases in [CrP] in Figure 1A have been fitted to a single exponential $y = y_0 + Ae^{-k/t}$. The calculated data points are in the larger open symbols and the fitted exponential in smaller solid symbols. The constants yielding the best fit to a single exponential are given in Table 2 for both Figure 1A and 1B.

**Figure 2. Correlation of the predicted increase in [CrP] and decrease in [Pi] with the decrease in the rate of ATP synthesis.** The predicted increase in [CrP] and decrease in [Pi] following work stoppage are shown for cytochrome c concentrations of 8, 16, and 32 μM. The conditions used are the same as those for Figure 1A: a creatine pool of 46 mM, an [NAD$^+$]/[NADH] of 0.33, and a pO$_2$ of 25 torr. The [CrP] scale is shown on the bottom abscissa while that for [Pi] is shown on the top abscissa. The scales have been adjusted so that the individual points superimpose. There is a nearly linear correlation between the [CrP] and [Pi] and the rate of ATP synthesis with the slope increasing with increasing cytochrome c concentration.

**Figure 3. Dependence of metabolism during the work to rest transition on the size of the creatine pool.** The [CrP] and oxygen consumption rates were calculated for muscle in which the creatine pool was 23 mM instead of the 46 mM used in the previous figures. The intramitochondrial [NAD$^+$]/[NADH], pO$_2$, and cytochrome c concentrations were 0.1, 25 torr, 16 μM, respectively. Two different work rates (0.6 and 0.3 mM ATP/sec) are presented. The increase in [CrP] and decrease in the rate of ATP production occur over a shorter time scale than for a creatine pool of 46 mM. The decrease in inorganic phosphate concentration for a pool of 23 mM is less than for a pool of 46 mM, so the [CrP]/[Cr] in the working steady state is lower.

**Figure 4A,B. The dependence of the rest to work metabolic transition on intramitochondrial [NAD$^+$]/[NADH].** The intramitochondrial NAD pool is the primary source of the reducing equivalents used by oxidative phosphorylation to synthesize ATP. The influence of the intramitochondrial [NAD$^+$]/[NADH] on the rest to work transition has been calculate for values of 0.1, 0.3, 1 and 3 while holding the creatine pool at 46 mM, the pO$_2$ at 25 torr, cytochrome c concentration at 16 μM. The predicted rates of ATP synthesis are plotted against [CrP]. As [CrP] increases from the working steady state to the about 34 mM in the resting state, the rate of ATP synthesis initially falls linearly with increase in [CrP] and decrease in [Pi]. There is a strong curvature at low rates, however, as the [CrP] and [Pi] asymptotically approach the resting steady state values. This is more obvious in Figure 4B where the y axis has been expanded 10 x in order to show the approach to the resting steady state in more detail. Decreasing intramitochondrial [NAD$^+$]/[NADH] decreases the [CrP] for any specific rate of ATP synthesis. For a rate of 0.6 mM ATP/sec, for example, the [CrP] in the working steady state is 24, 21.6, 19.2, and 16.4 mM for [NAD$^+$]/[NADH] values of 0.1, 0.3, 1, and 3, respectively.
Figure 5. The pO₂ dependence of metabolism during the rest to work transition. The changes during the rest to work transition were calculated for pO₂ values of 5, 10, 25, and 50 torr, when the creatine pool, intramitochondrial [NAD⁺]/[NADH], cytochrome c concentration, and work rate are 23 mM, 0.1, 16 μM, and 0.6 mM ATP/sec, respectively. The initial rate of increase in [CrP] and decrease in the rate of ATP synthesis after stopping work are independent of pO₂. The working steady state [CrP] is, however, lower for lower values of pO₂, 7.8, 8.9, 10.3, and 11 mM for pO₂ values of 5, 10, 25, and 50 μM, respectively. The time required to reach the resting steady state increases markedly with decrease in pO₂.

Figure 6. The amount of oxygen in muscle that can be used for each decrease of 1 torr in oxygen pressure. The muscle was assumed to have a myoglobin content of 0.5 mM and having a P50 for binding oxygen of 3.6 torr. The solubility of oxygen in cells was assumed to be 1.4 nmole/ml/torr (no hemoglobin, open squares). The oxygen released from oxymyoglobin becomes approximately 1.4 nmole/ml/torr at 30 torr and this increases rapidly as pO₂ falls below 30 torr (+ myoglobin, solid squares).

Figure 7. The increase in intramitochondrial [NADH] needed to maintain a constant rate of ATP synthesis (oxygen consumption) as the pO₂ is decreased from 50 torr to 5 torr. The model was used to calculate how much more reduced the intramitochondrial NAD couple needs to become in order to hold the rate of cytochrome c turnover (rate of ATP synthesis) constant at 2, 6, and 8 sec⁻¹ while the pO₂ was decreased from 50 to 5 torr. The [CrP], [Cr], and [Pi] was held constant at 28, 18, and 9 mM, respectively for an energy state of 2.4 x 10⁴ M⁻¹. The values for the [NADH] are shown as percent of total NAD⁺ and NADH. These turnover numbers are similar to those seen in unstimulated cultured cells or isolated hepatocytes. At constant energy state the intramitochondrial NAD couple has to be more reduced to support higher rates of ATP synthesis.

Figure 8. The decrease in energy state needed to maintain a constant rate of ATP synthesis as the pO₂ is lowered from 50 torr to 5 torr at constant [NAD⁺]/[NADH]. The dependence on energy state was calculated for cytochrome c TNs of 2/sec, 5/sec, and 10/sec and plotted as the [CrP] against pO₂. The creatine pool was 46 mM and the intramitochondrial [NAD⁺]/[NADH] was held constant at 0.2. As the pO₂ was lowered from 50 to 3 torr the decrease [CrP] decreases as needed to maintain the cytochrome c turnover constant.
Table 1. The measured time constants for increase in [CrP] and decrease in [Pi] in human muscle that were measured when exercise was stopped.

<table>
<thead>
<tr>
<th>Source</th>
<th>Muscle group</th>
<th>N</th>
<th>pH</th>
<th>TCrP-off</th>
<th>TPi-off</th>
</tr>
</thead>
<tbody>
<tr>
<td>McCann et al. 1995 (36)</td>
<td>Wrist Flexor</td>
<td>4</td>
<td>7.10</td>
<td>35</td>
<td>32</td>
</tr>
<tr>
<td>Marsh et al. 1993 (34)</td>
<td>Wrist Flexor</td>
<td>5</td>
<td>7.05</td>
<td>30.4</td>
<td>30.5</td>
</tr>
<tr>
<td>Barstow et al. 1994 (3)</td>
<td>Plantor flexors</td>
<td>5</td>
<td>6.98</td>
<td>26.3 ± 17.3</td>
<td>30.7 ± 22.5</td>
</tr>
<tr>
<td>McCully et al. 1994 (39)</td>
<td>Plantor flexors</td>
<td>4</td>
<td>6.99</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>McCreary et al. 1996 (37)</td>
<td>“ “ “ pH adj</td>
<td></td>
<td></td>
<td>33.3</td>
<td>28.6</td>
</tr>
<tr>
<td>Haseler et al. 1999 (19)</td>
<td>Plantar Flexion</td>
<td>6</td>
<td>7.08</td>
<td>25.0 ± 2.7</td>
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</tr>
<tr>
<td>Ryan et al. 2013 (48)</td>
<td>Plantar Flexion</td>
<td>16</td>
<td>7.01</td>
<td>31.5 ± 8.5</td>
<td></td>
</tr>
<tr>
<td>Holliss et al. 2013 (26)</td>
<td>Quadriceps</td>
<td>9</td>
<td>7.0</td>
<td>20.9 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>Vanhatalo et al. 2011 (50)</td>
<td>Quadriceps</td>
<td>9</td>
<td>7.0</td>
<td>23 ± 5</td>
<td></td>
</tr>
<tr>
<td>Vanhatalo et al. 2014 (51)</td>
<td>Quadriceps</td>
<td>8</td>
<td>7.11</td>
<td>24 ±4</td>
<td></td>
</tr>
<tr>
<td>Fulford et al. 2013 (12)</td>
<td>Quadriceps</td>
<td>8</td>
<td>7.03</td>
<td>25.4 ± 5.0</td>
<td></td>
</tr>
</tbody>
</table>

The data are all for exercise work rates and periods of time for which the muscle was not significantly acidified. This was done to avoid the effects of muscle fatigue and altered tissue pH. TCrP-off and TPi-off are the time constants in seconds for the increase in [CrP] and decrease in [Pi] obtained by fit of the measurements to a single exponential.
Table 2. The time course for the increase in [CrP] predicted by the model, as fitted to a single exponential.

<table>
<thead>
<tr>
<th>0.6 mM ATP/sec</th>
<th>yo (mM)</th>
<th>A</th>
<th>T (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyt c =8 μM</td>
<td>28.6</td>
<td>-12.6</td>
<td>22.7</td>
</tr>
<tr>
<td>Cyt c =16 μM</td>
<td>29.3</td>
<td>-7.5</td>
<td>14.3</td>
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</table>

<table>
<thead>
<tr>
<th>0.3 mM ATP/sec</th>
<th>yo (mM)</th>
<th>A</th>
<th>T (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyt c =8 μM</td>
<td>28.7</td>
<td>-7.1</td>
<td>24.3</td>
</tr>
<tr>
<td>Cyt c =16 μM</td>
<td>29.6</td>
<td>-4.6</td>
<td>16.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>0.15 mM ATP/sec</th>
<th>yo (mM)</th>
<th>A</th>
<th>T (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyt c =8 μM</td>
<td>29.2</td>
<td>-4.2</td>
<td>29.2</td>
</tr>
<tr>
<td>Cyt c =16 μM</td>
<td>29.8</td>
<td>-2.9</td>
<td>20.9</td>
</tr>
</tbody>
</table>

Predicted behavior was calculated for a creatine pool, cytochrome c concentration, pO₂, and [NAD⁺]/[NADH] of 46 mM, 8 or 16 μM, 25 torr, and 0.33, respectively. The concentration of inorganic phosphate was assumed to be 3 mM when the [CrP] was 34 mM. The data for work rates of 0.6, 0.3, and 0.15 mM ATP/sec were fitted to the equation \( y = y_0 + Ae^{-kt} \) using Origin. The values in the table are the best fit (least squares) to a single exponential. The increase in the time constant (T) as the steady state work rate decreases shows that the increase in [CrP] is not exponential. The deviation from exponential is small enough, however, that in experiments it would be “hidden” by the noise.