Control of respiration and bioenergetics during muscle contraction

Youngran Chung,1 Paul A. Molé,2† Napapon Sailasuta,3 Tuan Khanh Tran,1 Ralph Hurd,3 and Thomas Jue1

Departments of 1Biological Chemistry and 2Exercise Science, University of California, Davis; and 3GE Medical Systems, Fremont, California

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Chung, Youngran, Paul A. Molé, Napapon Sailasuta, Tuan Khanh Tran, Ralph Hurd, and Thomas Jue. Control of respiration and bioenergetics during muscle contraction. Am J Physiol Cell Physiol 288:C730–C738, 2005. First published November 10, 2004; doi:10.1152/ajpcell.00138.2004.—1H-NMR experiments have determined intracellular O2 consumption (VO2) with oxymyoglobin (MbO2) desaturation kinetics in human calf muscle during plantar flexion exercise at 0.75, 0.92, and 1.17 Hz with a constant load. At the onset of muscle contraction, myoglobin (Mb) desaturates rapidly. The desaturation rate constant of MbO2 in resting muscle, implying that the resting myoglobin; nuclear magnetic resonance; glycogen; oxygen; exercise

myoglobin; nuclear magnetic resonance; glycogen; oxygen; exercise

O2 consumption (VO2) in vivo depends on a dynamic interplay between metabolic demand and O2 supply (22). Muscle contraction demands energy and alters cellular metabolism to meet increased needs. It also stimulates O2 delivery through ventilatory cardiovascular peripheral blood flow and diffusion adjustments. How the cell mobilizes metabolic resources and triggers enhanced O2 delivery presents a central question in the study of respiratory control. Unfortunately, current techniques, such as indirect calorimetry, near-infrared spectroscopy (NIRS), and arteriovenous measurement, have not resolved this question definitively, because they can neither determine confidently the intracellular VO2 nor map the dependence of VO2 on ADP and available O2, especially at the onset of contraction in blood-perfused muscle (40, 42). From the vantage point of the current kinetic models of respiratory control, ADP or a limiting substrate regulates respiration (7). The model predicts that the product of ATP hydrolysis stimulates respiration to meet enhanced energy needs. In vivo myocardial studies, however, have failed to detect direct VO2 dependence on ADP (1, 23, 33). Although VO2 rises, the ADP level remains constant. In contrast, skeletal muscle shows an ADP-dependent VO2 (2). These experiments, however, relied on whole body VO2 or arteriovenous O2 difference measurements to extrapolate the pertinent intracellular VO2. Quite clearly, the presence of myoglobin (Mb) and the numerous mediating diffusion steps from the vasculature to the mitochondria raise questions about the accuracy of the extrapolated intracellular VO2 value, on which all quantitative analysis must critically depend. Without a definitive intracellular VO2 value, the dependence of VO2 on ADP remains in question. Nevertheless, the apparent difference in myocardial and skeletal muscle VO2 regulation suggests that ADP regulates VO2 differently in skeletal and myocardial muscle. Each skeletal muscle contraction cycle can certainly elicit a significant fluctuation in ADP (10). Some researchers, however, have downplayed any significant role for a metabolic regulation of VO2 and have pointed to experiments showing a tight match between O2 supply and VO2 demand in blood-perfused muscle (51).

1H-NMR studies have presented an approach to mapping the intracellular PO2 with the Mb signals in vivo (28, 34, 38, 50). These NMR studies have reported fully saturated oxymyoglobin (MbO2) in resting muscle, implying that the resting myocyte PO2 is well above Mb P50 of 2.93 mmHg [PO2 necessary to obtain 50% O2 saturation of Mb (P50) at 39°C] and that cytochrome oxidase is most likely saturated (6). Raising only the cellular PO2 to increase the cytochrome oxidase activity cannot directly enhance VO2 during muscle contraction. In fact, human gastrocnemius muscle studies have shown a decrease, not an increase, in PO2 during exercise, in agreement with the cryosection analysis of canine gracilis muscle (17, 38). Although the fall in cellular PO2 increases the O2 gradient from the capillary to the cell, O2 delivery from the cytosol to the mitochondria must increase to accommodate the increasing VO2 demand. A switch from free O2 diffusion to Mb-facilitated diffusion could serve as the compensating mechanism to enhance O2 transport, except that tissues with inhibited Mb function or without Mb do not exhibit any apparent handicap in respiration (14, 16, 19).

† Deceased 20 April 2000.

Address for reprint requests and other correspondence: T. Jue, Dept. of Biological Chemistry, Univ. of California, Davis, Davis, CA 95616-8635 (E-mail: TJue@ucdavis.edu).

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Because MbO₂ constitutes an O₂ depot in the cell, the MbO₂ signal, along with the Mb in vitro O₂ binding constant, provides a means of determining the intracellular PO₂. Moreover, its desaturation kinetics would reflect the intracellular VO₂, given that Mb can also deliver O₂ from the sarclemma to the mitochondria. If blood flow can deliver O₂ to match precisely the VO₂ need, then MbO₂ should not desaturate. Any rise in the ¹H-NMR proximal histidyl N₉₂₅ signal intensity of deoxymyoglobin (deoxy-Mb), which increases as cellular O₂ falls, indicates a mismatch between O₂ delivery and demand. The Mb desaturation kinetics can then serve as an index of intracellular O₂ utilization, or VO₂ (32, 34). Such measurement of intracellular VO₂ overcomes the limitation with indirect calorimetry, vascular O₂ extraction, or NIRS techniques, which require key assumptions to extract the intracellular VO₂.

Indeed, during muscle contraction, Mb desaturates rapidly to a steady state with a time constant (τ) of ~30 s. Although Mb deoxygenates to different steady-state levels as a function of workload, the kinetics time constant remains unchanged. Eadie-Hofstee kinetic analysis of the Mb-derived intracellular VO₂ vs. ADP reveals no ADP-dependent regulation of intracellular VO₂ at the onset of muscle contraction. After Mb desaturation has reached a steady-state level, however, VO₂ continues to rise. In this second phase of respiration, VO₂ does show a strong dependence on ADP, consistent with observations reported in the literature (2).

Intracellular VO₂ provides insight into the contribution of oxidative phosphorylation during a muscle contraction cycle. Given the reported value of 0.15 mM ATP per contraction in human gastrocnemius muscle, on the basis of intracellular VO₂, oxidative phosphorylation provides 36% of the energy (4). The result stands in sharp contrast to the orthodox view of muscle bioenergetics, which ascribes insignificant oxidative ATP production to any contraction cycle. However, Chung et al. (10) have raised questions about the low ATP turnover per contraction cycle and have presented experimental evidence to show that in each contraction, muscle consumes 3 mM ATP within milliseconds. At 3 mM ATP per contraction, the intracellular VO₂ can supply only ~2% of the energy cost. The fractional contribution of VO₂ to the overall energy demand, the large demand for ATP per contraction, and the need to sustain contraction for a prolonged period thus require the presence of a dynamic energy utilization-restoration cycle, such as the one proposed in the glycogen shunt theory (46). Indeed, using steady-state VO₂ as an estimate of the total ATP cost leads to an estimate of the glycogen contribution. The nonoxidative contribution from glycolysis represents ~31% of the energy demand during a muscle contraction.

The present study has established an approach to measurement of intracellular VO₂; has assessed the temporal interplay between metabolic demand, respiration, and hemodynamics; has identified an initial mismatch between O₂ supply and demand; has provided evidence for ADP-dependent and ADP-independent VO₂ in the context of current respiratory control models; has implicated a role for Mb in filling transient O₂ needs; has quantitated the significant contribution of oxidative phosphorylation to the energy demand; and has implicated glycolysis in the dynamic energy restoration-use cycle lasting for only milliseconds.

**MATERIALS AND METHODS**

**Experimental design.** The protocol for this study was reviewed and approved by the Human Subjects Welfare Committee of the University of California, Davis, as described in a previous report (38). Four young adult men were recruited from the student body of the university. All were untrained volunteers who gave written consent to participate in this study. They were informed of the procedures, requirements, and risks of participation in the study. Each individual attended the laboratory several times to practice plantar flexion exercise and to become familiar with breathing through the respiratory apparatus. During this time, the exercise intensity was adjusted so that the participant could reach peak O₂ uptake (VO₂peak).

Two sessions were required to complete this study. Session I was conducted in the Human Performance Laboratory and involved characterization of each participant’s body composition, steady-state VO₂ at several intensities of plantar flexion exercise. A fiberglass cast (ScotchCast; 3M, Minneapolis, MN) was then formed from below the knee to the ankle. The cast was used to estimate the leg volume and as a means to calibrate the percent desaturation of the deoxy-Mb signal. Session II used an identical protocol and involved duplicate determination of metabolic phosphates using ³¹P-NMR and of deoxy-Mb using ¹H-NMR during plantar flexion exercise (38, 50).

**Plantar flexion ergometer.** The ergometer consisted of a three-sided box of dimensions 25.4-cm width, 25.4-cm height, and 91.4-cm length, with a foot pedal on an axle at one end and a movable backplate at the other end. Latex rubber tubing (1.3-cm diameter, 34.3 cm²) with a Hooke’s constant of 31.12 N/cm length change was attached to the backplate and the axle of the foot pedal. Resistance to plantar flexion can be varied by the number of tubes used and/or by changing the stretch of tubing between the axle and backplate. The mechanical work of plantar flexion involved moving the pedal against a specified resistance across an arc of 3.8 cm. The pedal movement was controlled by stops for forward and reverse movements with plantar flexion and relaxation. In this study, power was incremented by varying the contraction from 45–55 to 70 repetitions per minute (RPM) or between 0.75–1.17 Hz. The resistance and the plantar flexion arc were held constant.

**Steady-state and peak VO₂ for plantar flexion exercise.** Indirect calorimetry first determined the energy expenditure. After resting for 10 min in a supine position, the participant breathed for 5 min through a mouthpiece and tubing connected to a Sensor Medics metabolic cart (model 2900; Sensor Medics, Anaheim, CA) for breath-by-breath determination of resting VO₂ and CO₂ production (VCO₂). Next, the participant performed a series of three to five exercise bouts at progressively higher intensity by varying the frequency from 45 to 70 RPM on the foot ergometer. The resistance remained constant. Each bout lasted 3 min and was followed by 6 min of rest. The calculated mean value during the last 30 s of each bout characterized the VO₂, VCO₂, and respiratory exchange ratio.

After a resting period of 10–15 min, the individual’s VO₂peak was determined by holding the resistance constant and progressively increasing the contraction frequency each minute until the participant could no longer maintain the required cadence. VO₂ and VCO₂ were determined throughout the test as described above. VO₂ was averaged over each 15-s interval. The highest VO₂ was designated as the participant’s VO₂peak.

**¹H-NMR and ³¹P-NMR experiments.** NMR measurements were performed on a 1-m bore diameter GE Sigma scanner (GE Medical Systems, Fremont, CA) at 1.5 T. The participant’s calf muscle was centered on top of a 5-inch-diameter surface coil and was strapped down with Velcro. ¹H-NMR (63.86 MHz) signal acquisition used a body coil transmit-surface coil receive configuration. Magnetic field shimming used a three-point Dixon method to improve the field homogeneity, yielding a water line width of ~40 Hz (45).

A selective excitation pulse sequence was optimized to excite the deoxy-Mb and deoxy-Hb His-F₈ proximal histidyl N₉₂₅ signals ~4.6 times.
kHHz from the water resonance (50). Numerical simulation and spectroscopic experiments verified that the experimental pulse length of 800 μs had a full width at half-maximum excitation of 2 kHz. At an offset of 800 Hz or 13 parts per million (ppm) from the excitation maximum, the pulse power dropped by 25%. For the steady-state measurements, the averaging of 200 free induction decays yielded the final signal, requiring 45 s of signal averaging. The repetition time was 160 ms. The spectral width was 16 kHz, and the data block size was 512. For the transient Mb desaturation experiments, acquiring the signals for a 20-s spectrum yielded sufficient signal to noise to follow the dynamic change in cellular O2 during contraction. All spectra were referenced to the water signal as 4.60 ppm at 39°C, which in turn was calibrated against 3-(trimethylsilyl)propionic acid-d4 sodium salt as 0 ppm.

31P-NMR signal intensities were normalized to the total signal following equation:

\[ \text{signal intensity} = \frac{\text{signal area}}{\text{total signal area}} \]

where \( \text{signal area} \) is the area under the peak, \( \text{total signal area} \) is the sum of all signal areas, and \( \text{total signal area} \) is the sum of all signal areas. 31P-NMR spectra revealed a slower fall in PCr and a slower rise in Pi (Fig. 2). Within 30 s, the PCr signal intensity changes much more slowly than does the Mb signal intensity. The PCr and Mb kinetics do not coincide.

Intracellular pH was calculated from the Pi signal using the following equation:

\[ \text{pH} = \text{pK} + \log \left( \frac{\delta_\lambda - \delta_0}{\delta_0 - \delta_\lambda} \right) \]

where \( \text{pK} = 6.9, \delta_\lambda = \delta_{\text{PPM}} \text{of} \left[\text{H}_3\text{PO}_4 \right]^- \text{at} 3.290 \text{ppm}, \delta_0 = \delta_{\text{PPM}} \text{of} \left[\text{HPO}_4^{2-}\right]^- \text{at} 5.805 \text{ppm}, \text{and} \delta_0 = \delta_{\text{PPM}} \text{of} \text{Pi} \text{referred to} \text{PCr} \delta_{\text{PPM}} \text{as} 0 \text{ppm} \) (9). PCr, Pi, and ATP levels were determined on the basis of integrated areas of PCr, Pi, and β-ATP signals, respectively. The ADP level was derived from 31P-NMR parameters and the creatine kinase (CK) equilibrium constant of 1.66 × 109 M⁻¹ (35). In determining the transient level of ADP, the analysis assumes that the near-equilibrium CK reaction permits the approximation of the instantaneous steady-state value with the equilibrated value as previously discussed (10). The spectroscopic data were exported from the Sigma system to a Sun Microsystems Sparc2 workstation and processed using Omega 6.0 software package.

**Intracellular PO2 and VO2**

Steady-state intracellular PO2 values were calculated from the following relationship:

\[ \text{PO2} = \frac{Y}{1 - Y} [\text{PO2}]_{50} \]

where \([\text{PO2}]_{50}\) is the partial O2 pressure required to half-saturate Mb and

\[ Y = \frac{\text{MbO}_2}{\text{Mb} + \text{MbO}_2} \]

A [PO2]_{50} of 2.93 mmHg was used for the calculation, assuming a muscle temperature of 39°C (43).

The intracellular VO2 calculation assumed that the initial rate of Mb desaturation arose from the cellular O2 demand at the beginning of muscle contraction. Mb served as the only O2 source. Differentiating the fitted curve of the experimental data showing the change in deoxy-Mb concentration vs. time \([y = c - c \cdot \exp(-x/t)]\) and evaluating the initial rate at \(t = 0\) yielded the initial rate of dMb/dt. The derivation of the dO2/dt assumed Mb concentration of 0.4 mM. Mb desaturation kinetics during a rapid cuffing of blood flow established the resting VO2 level.

**RESULTS**

Figure 1 shows the dynamic changes in the \(^1H\)-NMR and \(^31\text{P}\)-NMR spectra in human calf muscle during exercise at 70 RPM. In the resting state, no deoxy-Mb proximal histidyl N\(\cdot\)H signal appears in the \(^1H\)-NMR spectra and the cellular PO2 is sufficient to saturate Mb (Fig. 1A). The onset of exercise triggers a rapid Mb desaturation (Fig. 1). At all workload levels, MbO2 desaturates with \(\tau = 30\) s. The corresponding \(^31\text{P}\)-NMR spectra revealed a slower fall in PCr and a slower rise in Pi (Fig. 2). Within 30 s, the PCr signal intensity changes much more slowly than does the Mb signal intensity. The PCr and Mb kinetics do not coincide.

Figure 3A graphs the dynamic response of deoxy-Mb, PCr, ADP, and whole body VO2 levels during the beginning of muscle contraction at 70 RPM. Muscle contraction triggers MbO2 desaturation and the appearance of the deoxy-Mb prox-
imal histidyl N\(_2\)H signal. An exponential line fit of the deoxy-Mb kinetics reveals \(\tau = 29.9 \pm 7.3\) s. At 45 and 55 RPM, the \(\tau\) values do not change significantly (24.0 \(\pm\) 7.1 s and 27.4 \(\pm\) 6.3 s, respectively). The PCr level, however, changes more slowly. PCr decreases with \(\tau = 68.9 \pm 13.6\) s, consistent with previous reports in the literature (39). At 45 and 55 RPM, the PCr kinetics yielded \(\tau\) values of 60.4 \(\pm\) 14.2 s and 72.4 \(\pm\) 7.7 s, respectively. Whole body \(\dot{V}_O_2\) also changes more slowly than did Mbo\(_2\) desaturation. At 70 RPM, the \(\dot{V}_O_2\) is \(105.4 \pm 13.0\) s. At 45 and 55 RPM, the \(\dot{V}_O_2\) values are 50.3 \(\pm\) 14.0 s and 79.2 \(\pm\) 12.6 s, respectively. The ADP level as derived from the CK reaction rises with \(\tau = 84.1 \pm 28.1\) s at 70 RPM. At 45 and 55 RPM, the values are \(\tau = 33.4 \pm 10.1\) s and 71.5 \(\pm\) 17.9 s, respectively. The dynamic response of ADP and whole body \(\dot{V}_O_2\) to muscle contraction lags the kinetics of Mbo\(_2\) desaturation.

Figure 3B shows the changes in ATP, P\(_i\), and pH. Under work conditions, ATP level shows no significant dynamic or steady-state change. As the PCr level falls, however, P\(_i\) increases with kinetic \(\tau\) ranging from 45.6 \(\pm\) 7.9 s to 57.1 \(\pm\) 10.7 s (38, 52). The chemical shift of the P\(_i\) peak reflects the cellular pH and shows an initial 80 s of alkalinization arising from PCr hydrolysis. The P\(_i\) signal also exhibits a slight broadening, reflecting the pH heterogeneity from different muscle fibers. The data are presented in Table 1.

Figure 4A plots the steady-state whole body \(\dot{V}_O_2\) as a function of the intracellular P\(_O_2\) and ADP levels. As \(\dot{V}_O_2\) rises with increasing workload, intracellular P\(_O_2\) falls. ADP level, however, increases proportionally with \(\dot{V}_O_2\) (38). The Eadie-Hofstee plot in Fig. 4B shows the relationship between intracellular \(\dot{V}_O_2\) and ADP at the initiation of contraction. The intracellular \(\dot{V}_O_2\) levels correspond to 7.54 \(\mu\)M s\(^{-1}\) (45 RPM), 7.48 \(\mu\)M s\(^{-1}\) (55 RPM), and 8.94 \(\mu\)M s\(^{-1}\) (70 RPM). The analysis of intracellular \(\dot{V}_O_2\) as derived from the Mbo\(_2\) desaturation kinetics against ADP evaluated at \(t = 1\) s reveals a linear relationship, \(y = 0.09 \times -0.01\) (\(R = 0.99\)). Because Eadie...
Table 1. Exponential time constants for metabolite changes at different exercise intensities

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>45 RPM</th>
<th>55 RPM</th>
<th>70 RPM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>τ, s</td>
<td>R</td>
<td>τ, s</td>
</tr>
<tr>
<td>PCr</td>
<td>60.4±14.2</td>
<td>0.99</td>
<td>72.4±7.7</td>
</tr>
<tr>
<td>P_{i}</td>
<td>57.1±10.7</td>
<td>0.99</td>
<td>45.6±7.9</td>
</tr>
<tr>
<td>ADP</td>
<td>33.4±10.1</td>
<td>0.98</td>
<td>71.5±17.9</td>
</tr>
<tr>
<td>VO_{2,mb}</td>
<td>50.3±14.0</td>
<td>0.87</td>
<td>79.2±12.6</td>
</tr>
<tr>
<td>Deoxy-Mb</td>
<td>24.0±7.1</td>
<td>0.96</td>
<td>27.4±6.3</td>
</tr>
</tbody>
</table>

Nonlinear curve fitting of the time course data points to a monoexponential function \( y = a + (c - a) \exp^{-x/t} \), where \( y \) represents the metabolite level, \( x \) denotes time in seconds, yields the exponential time constant \( (\tau) \) expressed in seconds. Correlation coefficients \( (R) \) show how well the exponential curve fits the raw data. RPM, repetitions per minute; PCr, phosphocreatine; VO_{2,mb}, oxygen consumption; Deoxy-Mb, deoxymyoglobin.

Hostee analysis requires any ADP-dependent VO_{2} to show a negative, not a positive, slope; the results indicate that the increase in VO_{2} does not depend on ADP at the initiation of muscle contraction.

DISCUSSION

Intracellular VO_{2}. At the beginning of muscle contraction, Mb desaturates rapidly with an exponential time constant of 30 s. As contraction frequency increases to 45, 55, and 70 RPM, the steady-state level of Mb desaturation increases from 0 to 30%, 36%, and 48%, respectively, reflecting a progressive drop in intracellular PO_{2} and an enhanced O_{2} gradient from the vasculature to the cell (38). Although final Mb desaturation level increases with workload, the kinetic time constant for Mb desaturation for each respective state does not deviate significantly.

The desaturation kinetics of MbO_{2} implies that the cell has rapidly increased its O_{2} demand, which presumably reflects the change in intracellular VO_{2}. Such an assumption appears reasonable, given the primary role of Mb either as the predominant cellular O_{2} store or as a facilitator of intracellular O_{2} transport and source of O_{2} to satisfy the increased demand for O_{2} during muscle contraction (55). If the O_{2} supply from blood flow and in the interstitium does not contribute significantly at the onset of contraction, then the rate of Mb desaturation reflects the change in intracellular VO_{2}. Although the current NMR detection sensitivity cannot measure the Mb signal with finer time resolution, the data nevertheless present a lower limit estimate because the analysis presuming no contribution of free cellular or vasculature O_{2} until Mb desaturation reaches a steady state.

Given the cellular Mb concentration of ~0.4 mM in human gastrocnemius muscle and a resting VO_{2} of 2.02 \( \mu \)M s\(^{-1}\), dMb/dt yields intracellular VO_{2} ranging from 7.5 to 8.9 \( \mu \)M s\(^{-1}\) at 45–70 RPM (4). The observed VO_{2} is within the range of the NIRS-determined VO_{2} of 3.0–31.2 \( \mu \)M s\(^{-1}\) in forearm muscle during isometric handgrip exercise (42, 50). NIRS, however, cannot discriminate intracellular vs. vascular change in VO_{2}.

The initial, rapid change in Mb desaturation reflects the intracellular VO_{2} and appears to correspond to the initial phase observed in pulmonary O_{2} uptake experiments (54). Indeed, recent pulmonary O_{2} uptake measurements have extrapolated an initial 30-s O_{2} uptake phase consistent with a distinct 20- to 40-s initial phase in the single-myocyte intracellular PO_{2} at the beginning of contraction (24, 54).

O_{2} supply and intracellular VO_{2}. The Mb desaturation kinetics yield insight into O_{2} utilization and delivery at the initiation of muscle contraction. Under resting conditions, the NMR spectra reveal no detectable deoxy-Mb signal from the proximal histidyl N\(_{\text{H}}\), even though this experimental technique can detect quantitatively the deoxy-Mb signal at ~10% deoxygenation in these calf muscle experiments. Given the in vitro Mb P_{50} of 2.93 mmHg at 39°C, the undetected deoxy-Mb signal in the resting state implies that the intracellular PO_{2} must saturate >90% of the Mb, or PO_{2} >12 mmHg. No apparent O_{2} limitation exists, then, in resting muscle. These results are in agreement with studies of in situ heart and skeletal muscle, which also have not revealed any detectable deoxy-Mb signal in the basal normoxic state (33, 37, 38, 56).

At the onset of exercise, the rapid deoxygenation of Mb implies a transient mismatch between O_{2} supply and demand.
Indeed, if O2 delivery matches precisely the respiration need in
human calf muscle, then Mb would not need to supply any O2
(3, 29). No Mb desaturation should occur. However, if a
transient mismatch exists, then the mitochondria draw from an
immediate intracellular O2 storage until convective flow or
conductive diffusion can respond to the change in intracellular
O2 demand.

Under such assumptions, the Mb desaturation kinetics thus
reflects intracellular VO2. This analysis assumes that all O2
originates from Mb. Free O2 does not contribute significantly
until Mb has reached its deoxygenated steady state within 30 s
and the vasculature has adapted its blood flow to deliver more
O2. Both the undefined O2 contribution and the 20-s signal
averaging of the Mb resonance suggest that Mb-derived intra-
cellular VO2 represents a low-limit estimate.

The O2 gradient in conductive diffusion cannot immediately
accommodate the sudden rise in VO2. As VO2 increases 273 to
343% above its resting level, the PO2 gradient from the vascula-
ture to the cell as reflected in the venous PO2 and the
Mb-derived intracellular PO2 changes only 11.3–16.3% (Table
2). The modest change in the PO2 concentration gradient and
the associated diffusion driving force cannot accommodate the
rise in VO2. As previously reported, the vasculature requires a
finite time, ~30 s, to adapt, increase flow, and decrease the
capillary-to-cell distance to restore the match between O2
supply and demand (20, 25). This viewpoint coincides with the
observed rapid Mb desaturation to a steady-state level. VO2,
however, continues to rise after Mb has desaturated to a
steady-state level. The temporal sequence appears to be as
follows. A cellular O2 supply-and-demand mismatch at the
onset of contraction uses the Mb oxygen store to provide the
immediate source for respiration. Convective-conductive dif-
fusion then restores the O2 supply-and-demand balance to
spare additional O2 loss from Mb and to allow VO2 to continue
rising (30).

Consistent with phase II of pulmonary O2 uptake, VO2
continues to increase after Mb desaturation has already reached
a steady state (40). From the vantage point of mass balance, the
diffusion analysis of phase II VO2 indicates that the enhanced
vasculature-to-cell O2 gradient, increased convective flow,
increased conductive diffusion, and increased flow of carbon
substrate into the tricarboxylic acid cycle now balance O2
supply and demand. Yet, from an enzyme kinetics vantage
point, increasing respiration and oxidative phosphorylation in
the face of a decreased O2 level as reflected in the Mb
saturation state poses a question about O2 as the rate-limiting
substrate in the cytochrome oxidase reaction under these
experimental conditions. In the current models of respiratory
control, if only the O2 supply regulates the reaction velocity
(i.e., VO2), then O2 must increase, not decrease, as VO2 rises (7,
12). Moreover, microscopic kinetic analysis of the cytochrome
oxidase reaction points consistently to proton translocation, not
O2 binding, as the kinetic rate-limiting step (5, 18).

Even if intracellular O2 increases with rising VO2, it can
regulate the cytochrome oxidase reaction only if the O2 resting
concentration is near the reaction Km. If intracellular O2 satu-
rates cytochrome oxidase, however, then any increase in O2
cannot affect the reaction velocity. In vivo experiments have
determined that, at rest, the intracellular O2 concentration in
the heart and skeletal muscle must exceed a PO2 of 12 mmHg,
well above the P50 of 2.93 mmHg at 39°C (33, 38). Otherwise,
NMR would detect a deoxy-Mb signal. In vitro enzyme stud-
ies, however, have ascribed a Km of 0.1 μM to cytochrome
oxidase (53). Such a wide difference in Km values suggests that
the cell must maintain a large 100:1 O2 gradient from the
sarcolemma to the mitochondria if O2 is at the Km
concentration range of the cytochrome oxidase reaction (8).
Although previous studies have reported such an intracellular
gradient, other experiments have not provided corroborative evidence
(9). No significant deviation in the linear relationship between
the physiological and/or metabolic indices and the percent Mb
saturation has appeared, as the gradient coherence model
would predict (6, 48). Moreover, a gradient that sets the O2 at
the cytochrome oxidase Km can regulate respiration only if the
O2 concentration at the mitochondrial level increases. The
experimental data, however, show an overall decreasing intra-
cellular PO2 level as whole body VO2 rises. Unless the large
cellular PO2 gradient collapses, the declining O2 level militates
against O2 as the sole determinant of VO2 during muscle
contraction.

Alternatively, if such an O2 gradient does not exist, then the
intracellular O2 saturates cytochrome oxidase under resting and
exercising conditions. Raising the intracellular or extracellular
PO2 cannot regulate the enzyme reaction velocity or respira-
tion. Indeed, recent Xenopus fiber studies have shown that
increasing the extracellular PO2 (PEO2) does not significantly
alter the intracellular PO2 (PI02) during stimulation (29).

**ADP-independent VO2 regulation at the initial phase of
contraction.** If the O2 supply does not regulate VO2 at the
initiation of contraction, then current respiratory control mod-
els would point to either ADP or NADH as the regulatory
factor cast as either an equilibrium or a kinetic model. A rising
level of either ADP or NADH, ADP/ATP, or NADH/NAD+
stimulates respiration, which in turn increases the reduction of
O2 by cytochrome oxidase. Because Mb desaturation kinetics
directly yield intracellular VO2, an Eadie-Hofstee kinetic anal-

Table 2. Initial VO2 at different work intensities

<table>
<thead>
<tr>
<th>RPM</th>
<th>Intracellular VO2, μM·s⁻¹</th>
<th>∆MbO2: Desaturation, Ref. 38</th>
<th>Intracellular PO2, mmHg</th>
<th>∆PO2: Capillary to Cell, mmHg</th>
<th>%∆PO2</th>
<th>%∆VO2</th>
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<tr>
<td>45</td>
<td>7.54</td>
<td>30.2</td>
<td>6.8</td>
<td>33.2</td>
<td>18.6</td>
<td>273</td>
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<tr>
<td>55</td>
<td>7.48</td>
<td>35.6</td>
<td>5.3</td>
<td>34.7</td>
<td>23.9</td>
<td>270</td>
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<td>70</td>
<td>8.94</td>
<td>48.4</td>
<td>3.1</td>
<td>36.9</td>
<td>31.8</td>
<td>343</td>
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<tr>
<td>Resting state</td>
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<td>12.0</td>
<td>28.0</td>
<td>0</td>
<td>0</td>
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d(O2)/dt is calculated at t = 0 s at the beginning of each exercise bout, assuming a 0.4 mM Mb concentration for human gastrocnemius muscle. The experimentally determined value includes a resting state VO2 component of 2.02 μM·s⁻¹ (derived from ∆MbO2/dt in response to rapid cuffing of blood flow). Conversion of micromolar values to millimeters of Hg involves the use of a scaling factor of 1 mmHg O2 = 1.34 μM dissolved O2. The resting intracellular PO2 is set at 12.0 mmHg, and the mean end capillary PO2 corresponds to 40 mmHg. ∆MbO2, change in oxymyoglobin; ∆PO2, change in oxygen tension; %∆VO2, %change in oxygen consumption.
ysis can readily assess any ADP-dependent rise in intracellular \(V_\text{O}_2\) at the start of muscle contraction. On the basis of the assumption of a near-equilibrium CK enzyme and the approximation of the instantaneous steady state with the equilibrium state, the Eadie-Hofstee analysis of \(V_\text{O}_2\) dependence on ADP reveals no discernible correlation (11). In fact, the data reveal a positive slope in the graph of \(V_\text{O}_2/\text{ADP} vs. \text{VO}_2\) when the Eadie-Hostee analysis demands a negative slope to show any substrate dependence in enzyme kinetics.

In phase II \(V_\text{O}_2\), however, the same kinetic analysis of ADP vs. \(V_\text{O}_2\) indicates a clear ADP-dependent regulation of \(V_\text{O}_2\), which is in excellent agreement with previous reports in the literature (2). The present experimental results suggest that the cell exerts biphasic control of respiration in which the initial phase appears to be ADP independent but the latter phase is ADP dependent.

\(V_\text{O}_2\)-dependent ATP production during muscle contraction.

From the vantage of cellular bioenergetics, any ADP control must still fit within a framework of energy fluxes, partitioned broadly into oxidative and nonoxidative components. The regulation of the respective fluxes through CK, glycolysis, glycogenolysis, and oxidative phosphorylation during muscle contraction forms a basis for the overall control of bioenergetics. On the basis of the Mb desaturation kinetics, the cell consumes 7.5–8.9 \(\mu\text{M}\) of \(O_2\) at the onset of contraction, consistent with a 30-s depletion of \(\text{MbO}_2\) to a steady-state level of 30–48% \(\text{MbO}_2\) saturation. Using the canonical ATP production rate per mole of \(O_2\) consumed (P:O) ratio of 3:1 yields the corresponding oxidative ATP production rate of 45–54 \(\mu\text{M} \cdot \text{s}^{-1}\) or \(\sim 50 \mu\text{M} \cdot \text{per contraction. Given the 0.36 mM (−0.3 mM) ATP g}^{-1}\) per contraction observed in freeze-clamp experiments with frog muscle, the initial oxidative ATP production can supply 17% of the ATP required for each contraction (27). In a recent experiment with human forearm muscle stimulated at 1 Hz, however, the investigators extrapolated from steady-state measurements a lower energy cost of 0.15 mM ATP per contraction (4). That study’s experimental condition corresponds closely to the present study’s exercise protocol at 70 RPM. Given ATP utilization at 1 Hz stimulation, \(V_\text{O}_2\) can actually fuel 36% of the required energy. ATP from oxidative metabolism no longer has a paltry role. Instead, oxidative phosphorylation supplies a significant fraction of the energy per muscle contraction. In contrast to the orthodox view of muscle bioenergetics, which ascribes an insignificant contribution of oxidative ATP production during a contraction cycle and underscores a predominant role for PCR as a transient energy buffer, the present analysis shows a significant 36% contribution to the currently accepted energy cost per twitch.

However, some questions surround the accuracy of the currently accepted ATP use during muscle contraction. The level of ATP use per twitch originates from freeze-clamp experiments or from extrapolation of the PCR kinetics with an averaged time resolution of minutes. With freeze-clamp techniques, the time resolution is only 100 ms, whereas the muscle contraction cycle peaks in ~20 ms. If a muscle contraction consumes much more ATP in (~100 ms, the freeze-clamp technique cannot accurately quantify the value (10). With the analysis of the gradual decline in PCR, the extrapolation of the energy cost per twitch from d[PCr]/dt at \(t = 0\) presumes an insignificant restoration of ATP from oxidative phosphorylation or other metabolic pathways (15). Clearly, the present report shows that oxidative phosphorylation does contribute significantly.

Indeed, Chung et al. (10) have presented a different picture of the twitch energy cost and have hypothesized a much larger energy fluctuation. They devised a novel NMR technique to measure millisecond changes in PCR during a muscle contraction cycle and observed ~3 mM ATP per contraction, which is ~10 times greater than the currently accepted energy cost (10). With 3 mM ATP, oxidative phosphorylation can supply only 2% of the energy per twitch. PCR would deplete rapidly unless an energy restoration process allows PCR to recover between contractions. Another source must fuel the millisecond energy bursts during each twitch and sustain continuing contractions.

A novel glycogen shunt theory has been proposed that glycogen supplies that energy (46). However, several dozen contractions would deplete the ~70 mM glucosyl unit of glycogen, which experiments have not observed. Oxidative phosphorylation must still replete the energy store in the steady state. Although \(V_\text{O}_2\) at the initiation of contraction cannot supply all of the ATP contraction need, \(V_\text{O}_2\) does rise as contraction proceeds, and the vasculature increases the \(O_2\) supply. The increased \(V_\text{O}_2\) can then provide additional oxidative ATP to replenish PCR and allow for glycogen resynthesis.

Implication for the role of \(\text{Mb}\) in supplying \(O_2\). The present report also casts a perspective on the role of \(\text{Mb}\) as a cellular \(O_2\) store. As a cellular \(O_2\) store, \(\text{Mb}\) sustains the myocardial aerobic demand for only a few contraction cycles. During global ischemia, \(\text{Mb}\) desaturates with a \(t_{1/2}\) of 0.9 min, consistent with the PCR depletion \(\tau\) value, and during postischemic reperfusion, \(O_2\) returns rapidly to control levels (9). The \(O_2\) store in \(\text{Mb}\) does not confer any significant advantage in the cellular response to ischemia or postischemic recovery as CO inhibition and other experiments have demonstrated (Ref. 9 and Huang S et al., unpublished manuscript). However, the rapid \(\text{MbO}_2\) desaturation observed in the present study suggests that \(\text{Mb}\) also has a significant role in the transient state. \(\text{Mb}\) appears to buffer the transient energy demand by providing an immediate source of \(O_2\) at the onset of muscle contraction. These observations stimulate the continuing discussion about the transient- and steady-state roles of \(\text{Mb}\) in mammalian tissue.

An alternative view of bioenergetics of muscle contraction. In the current respiratory control models, the analysis has focused on the influence of ADP, NADH, or \(O_2\) on \(V_\text{O}_2\) in either the kinetic or thermodynamic formulation, especially with respect to a rate-limiting substrate or enzyme activity. The analysis provides continuity between past and present studies but overlooks a fundamental issue: the tight interaction of cellular metabolite flux militates against a simple reduction of all enzyme reactions to a rate-limiting step. Indeed, metabolic control analysis has pointed out this weakness and has introduced control coefficient and elasticity terms to characterize metabolic flux (13).

From the perspective of metabolic control, the present study’s results provide a basis for partitioning the energy fluxes. At the onset of muscle contraction, the demand for ATP increases with cross-bridge movement. Given the assumption that at steady state oxidative phosphorylation provides the predominant source of energy, the whole body \(V_\text{O}_2\) of \(13.2 \pm 2.1 \text{ ml} \cdot \text{min}^{-1} \cdot 100 \text{ ml}^{-1}\) at 70 RPM reflects an ATP consumption rate of \(589 \mu\text{M} / \text{s} \cdot \text{min}^{-1}\). This energy consumption rate
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provides an estimate of the total energy demand at the onset of contraction, using the canonical 3:1 P:O ratio. The intracellular \( \text{V} \dot{\text{O}}_{2} \) as assessed on the basis of the Mb desaturation kinetics shows an ATP production rate of 54 \( \mu \text{M/s} \) (9% of the energy need); PCR supplies ATP at 357 \( \mu \text{M/s} \) (61% of the energy need). The missing energy (30% of the energy need) must then arise from anaerobic metabolism, most notably through glycolgenolysis or glycolysis. Isotope tracer studies, however, have indicated that exercising muscle consumes glucose at only 1.1 \( \mu \text{M/s} \), producing ATP at only 2.2 \( \mu \text{M/s} \) (31, 41). Consequently, glycolgenolysis must supply ATP energy at \( \sim 170 \mu \text{M/s} \), corresponding to a glycogen metabolism rate of 54 \( \mu \text{M/s} \). That rate of glycolgenolysis falls well within the value (120 \( \mu \text{M/s} \)) observed in contracting human muscle (47). Because the total muscle glycogen pool is \( \sim 70 \text{mM} \), glyco-
genolysis can sustain the missing energy need for well over 25 min.

Metabolic transient studies of rat muscle, however, have indicated that the ATP consumption rate is actually much higher, approaching 3 mM ATP/contraction or 3 mM/s, given a 1-Hz contraction rate (10). Although the muscle glyco
genolysis rate could exceed 170 \( \mu \text{M/s} \) to accommodate the increased ATP need, the energy demand rapidly depletes the total glycogen pool within 25 contractions, which experiments have not observed. The missing energy, given the transient energy demand, implies that the intracellular \( \text{V} \dot{\text{O}}_{2} \) as reflected in the Mb desaturation kinetics underestimates the actual \( \text{V} \dot{\text{O}}_{2} \).

An underestimate of \( \text{V} \dot{\text{O}}_{2} \) seems reasonable because the analysis relies on a 20-s time-averaged change of the de-
oxy-Mb proximal histidyl \( \text{N}_{2}\text{H} \) signal to extract the Mb de-
saturation rate per contraction or per second. Such an analysis parallels the experimental approach to determine the change in PCR per contraction from time-averaged signals. Extrapolating from the overall time-averaged PCR kinetics during muscle stimulation can lead to the change in PCR per contraction, provided that all ATP generation pathways contribute insignifi-
cantly. As the metabolic transient study has shown, that approach underestimates the PCR/contraction (10).

The analysis also presumes that \( \text{O}_{2} \) in the blood or in the interstitial space contributes insignificantly during the entire contraction-relaxation cycle. The actual contraction phase, however, represents only a small fraction of the overall cycle. During the relaxation phase, \( \text{O}_{2} \) can readily diffuse from the interstitium or the vasculature.

On the basis of these considerations, the synergistic interac-
tion of oxidative phosphorylation and glyco
genolysis regulates the metabolic flux to meet the enhanced energy demand at the onset of contraction. CK, as a near-equilibrium enzyme, would presumably exert no significant control. Additional experiments must now be conducted to determine the transient changes in glycogen and Mb and to quantify accurately the contribution of oxidative phosphorylation and glyco
genolysis in regulating energy flow during a muscle contraction cycle.

In conclusion, the present study has established a method of measuring intracellular \( \text{V} \dot{\text{O}}_{2} \) at the initiation of muscle contrac-
tion. Given the rise in intracellular \( \text{V} \dot{\text{O}}_{2} \), no significant rise in ADP has occurred as predicted by the kinetic model in the regulation of respiration. Moreover, the intracellular \( \text{O}_{2} \) level decreases rapidly. On the basis of the conventional analytical model, these observations suggest that \( \text{V} \dot{\text{O}}_{2} \) does not depend on \( \text{O}_{2} \) and ADP at the beginning of muscle contraction. Other regulators must intervene. Current respiration control theory points to an out-of-equilibrium, NADH-dependent reaction that modulates the redox state or the NADH level, which in turn stimulates respiration. Although PDH stimulation should increase \( \text{V} \dot{\text{O}}_{2} \), experiments enhancing PDH activity with di-
chloroacetate have yielded inconsistent results (21, 26, 33, 44, 49).

On the basis of the steady-state estimate of energy require-
ments during muscle contraction, the energy fluxes partition as follows: \( \text{V} \dot{\text{O}}_{2} \), 9%; PCR, 61%; glyco
genolysis, 30%; and glucose, negligible. Metabolic transient studies of rat muscle, however, have indicated that the ATP consumption rate is actually much higher, approaching 3 mM ATP/contraction or 3 mM/s, given a 1-Hz contraction rate (10). Glyco
genolysis and \( \text{V} \dot{\text{O}}_{2} \) thus must interact synergistically to provide energy during a muscle contraction cycle.

With the Mb-derived intracellular \( \text{V} \dot{\text{O}}_{2} \) measurement, the present results show an initial mismatch between \( \text{O}_{2} \) supply and demand, an ADP-dependent and -independent mode of respiratory control, a significant role for Mb in filling the transient \( \text{O}_{2}, \) need, a significant energy contribution from glyco
genolysis, and oxidative phosphorylation over a dynamic, millisecond-duration muscle contraction cycle.

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