Pyruvate and citric acid cycle carbon requirements in isolated skeletal muscle mitochondria

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Messer, Jeffrey I., Matthew R. Jackman, and Wayne T. Willis. Pyruvate and citric acid cycle carbon requirements in isolated skeletal muscle mitochondria. Am J Physiol Cell Physiol 286: C565–C572, 2004. First published November 5, 2003; 10.1152/ajpcell.00146.2003.—Carbohydrate depletion precipitates fatigue in skeletal muscle, but, because pyruvate provides both acetyl-CoA for mainline oxidation and anaplerotic carbon to the citric acid cycle (CAC), the mechanism remains obscure. Thus pyruvate and CAC kinetic parameters were independently quantified in mitochondria isolated from rat mixed skeletal muscle. Mitochondrial oxygen consumption rate (Jₒ) was measured polarographically while either pyruvate or malate was added stepwise in the presence of a saturating concentration of the other substrate. These substrate titrations were carried out across a physiological range of fixed extramitochondrial ATP free energy states (ΔGₒ), established with a creatine kinase energy clamp, and also at saturating [ADP]. The apparent Kₘ,malate for mitochondrial Jₒ ranged from 21 to 32 μM, and the apparent Kₘ,pyruvate ranged from 12 to 26 μM, with both substrate Kₘ values increasing as ΔGₒ declined. Vₘₕₐₓ for both substrates also increased as ΔGₒ fell, reflecting thermodynamic control of Jₒ. Reported in vivo skeletal muscle [malate] are >10-fold greater than the Kₘ,malate determined in this study. In marked contrast, the Kₘ,pyruvate determined is near the [pyruvate] reported in muscle approaching exhaustion associated with glycogen depletion. When data were evaluated in the context of a linear thermodynamic force-flow (ΔGₒ-Jₒ) relationship, the ΔGₒ-Jₒ slope was essentially insensitive to changes in [malate] in the range observed in vivo but decreased markedly with declining [pyruvate] across the physiological range. Mitochondrial respiration is particularly sensitive to variations in [pyruvate] in the physiological range. In contrast, physiological [malate] exerts very little, if any, influence on mitochondrial pyruvate oxidation measured in vitro.

bioenergetics; fatigue; anaplerosis; Krebs cycle; fuel limitation; metabolic control analysis

CARBOHYDRATE (CHO) is an important oxidative substrate and makes an increasingly critical contribution to the fuel supply of active muscle as exercise intensity is increased (29, 41). At exercise intensities within the moderate aerobic range (~60–75% VO₂ max), CHO depletion and fatigue often coincide (4, 13). However, the mechanisms underlying this association between CHO depletion and fatigue are not completely understood.

CHO not only serves as an important oxidative substrate but also provides anaplerotic support for the citric acid cycle (CAC) (36). Sahlin et al. (43) observed progressively diminishing CAC intermediate (CACI) levels in biopsies from working skeletal muscle during steady-state, submaximal exercise to exhaustion. In contrast, the acetylcaridine-to-carminine ratio remained stable, which was interpreted to indicate stable, and adequate, acetyl-CoA availability. On the basis of these findings, they proposed that a decline in CACI concentrations constitutes the primary event linking CHO depletion to fatigue. More specifically, they proposed that a limiting CACI pool precipitated a falling cellular energy status, which provided a compensatory activation of substrate-limited citrate cycle enzymes but also inhibited the rate of energy utilization by the contractile apparatus (43). Their insightful analysis therefore interpreted carbohydrate-limited muscular fatigue as being the consequence of the connectivity property of metabolic control analysis (7). The question that remained, however, was whether CACI concentrations actually fell to levels low enough to require compensatory CAC enzyme activation by kinetic and/or energetic (“thermokinetic”) signals.

Correlative evidence, which demonstrates comcomitance of events such as falling CACI and fatigue onset, is useful and important, but it cannot quantitatively evaluate the adequacy of CHO in support of anaplerotic vs. mainline oxidative roles (43, 46). Moreover, more recent work by Gibala and others (see Ref. 22 for review) has advanced the concept that the CACI pool of skeletal muscle responds to moderate aerobic exercise with what appears to be an excessive expansion. Traditionally, models of substrate adequacy relied on enzyme kinetic parameters such as the Michaelis constant (Kₘ) and maximal velocity (Vₘₕₐₓ) to determine whether a substrate is limiting. More recently, thermodynamic force-flow relationships (5, 33) and the concepts of metabolic control analysis (MCA) (7, 24) have been used to evaluate the impact of altered substrate levels on the control of metabolic flux.

Malate accounts for the majority of CACI carbon, is rapidly taken up, and provides oxaloacetate to the citrate synthase reaction to condense with pyruvate-derived acetyl-CoA in one high-Vₘₕₐₓ dehydrogenase step. For these reasons, we have used malate as the source of CACI carbon in these studies. In preliminary experiments we found that added malate is rapidly distributed throughout the CACI pool. We also observed that other CACI sources, such as 2-oxoglutarate and fumarate, also are distributed throughout the pool and led to the same general conclusions regarding CACI kinetics.

Accordingly, the purpose of the present study was to provide an evaluation of the distinct contributions of pyruvate and malate (CACI) availability to the support of oxidative energy production in isolated skeletal muscle mitochondria.

METHODS

Animal and muscle preparation. All procedures were in accordance with the guiding principles in the care and use of animals at Arizona State University. Quadriceps femoris and triceps surae muscle groups

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obtained from rats weighing 250–300 g served as the source of mixed skeletal muscle mitochondria. Animals were killed with an overdose of carbon dioxide. Hindquarters were quickly skinned, skeletal muscle was rapidly excised from each hindlimb, and the muscles were immediately placed in an ice-cold solution containing (in mM) 100 KCl, 40 Tris HCl, 10 Tris base, 5 MgCl₂, 1 EDTA, and 1 ATP, pH 7.40 (solution I) (38).

Isolation of mitochondria. Mitochondrial isolations were performed at 0–4°C according to the methods of Makinen and Lee (38). Excised muscles were trimmed to remove fat and connective tissues and were then minced, weighed, and placed in 9 volumes of solution I. Protease (Nagarse; Sigma Chemical, St. Louis, MO) was added (5 mg/g wet muscle), and the digested mince was minced continually for 7 min. Digestion was terminated through the addition of an equal volume of solution I, and the mince was homogenized with an Ultra-Turrax (Cincinnati, OH) blender for 15 s at 40% of full power.

The homogenate was centrifuged at 700 g for 10 min in a refrigerated centrifuge (model J2-21M/E; Beckman) to pellet down contractile protein and cellular debris. The supernatant was rapidly decanted through a double layer of cheesecloth and centrifuged at 14,000 g for 10 min to pellet down the mitochondrial fraction. The supernatant was discarded, the mitochondrial pellet was resuspended and washed in a volume equal to the original homogenate in a solution containing (in mM) 100 KCl, 40 Tris HCl, 10 Tris base, 1 MgSO₄, 0.1 EDTA, 0.2 ATP, and 2% (wt/vol) bovine serum albumin (BSA; Sigma Chemical, no. A-7030, fatty acid content <0.01%), pH 7.40 (solution II), and the suspension was centrifuged at 7,000 g for 10 min. The supernatant was discarded, and the pellet was resuspended in 20 ml of a solution similar to solution II but without BSA (solution III). This resuspended pellet was subsequently centrifuged at 3,500 g for 10 min.

The final mitochondrial pellet was suspended in 500–700 µl of a solution containing (in mM) 220 mannitol, 70 sucrose, 10 Tris-HCl, and 1 EGTA, pH 7.40, yielding a final protein content of 6.5–9.5 mg mitochondrial protein/ml (37).

Mitochondrial respiration. Mitochondrial oxygencodepletion (Jₜ) was measured polarographically in a respiration chamber maintained at 37°C (Rank Brothers, Cambridge, UK). Incubations were carried out in a 3.0-ml final volume of respiration medium adapted from Wanders et al. (50). For state 3 determinations, the medium contained (in mM) 100 KCl, 50 MOPS, 10 K₃PO₄, 10 MgCl₂, 1 EGTA, and 0.2% BSA, pH 7.00.

State 3 (maximal) respiration rates were initiated with a bolus addition of ADP, 0.67 mM final concentration, with 1 mM pyruvate plus 1 mM malate (P + M) as oxidative substrates. State 3 and state 4 (resting) respiration rates, as described by Estabrook (19), were measured, and the respiratory control ratio (RCR) was calculated as the ratio of state 3-to-state 4 respiration. The ADP/O ratio was also determined (19).

Steady-state respiration studies were performed with isolated mitochondria under three energetic states. A creatine kinase (CK) energy clamp was used to establish desired ATP/ADP ratios. Specifically, phosphocreatine-to-creatine (PCr/Cr) ratios were varied within a large and constant total creatine ([PCr] + [Cr] = 50 mM) pool in the presence of 5 mM ATP and 25 U/ml CK. The energy clamp forms and stabilizes the ATP/ADP ratio and, thus, the free [ADP], by utilizing the CK equilibrium (K₉Cr) (35):

\[
\frac{[ATP]}{[ADP]} = \frac{[PCr]}{[Cr]} \quad (1)
\]

where K₉Cr is 177 at 38°C, pH 7.0, and 1 mM free Mg²⁺ (23). In the presence of a fixed inorganic phosphate pool (10 mM), the energy clamp system exposes mitochondria to a fixed energetic environment with a Gibbs free energy of ATP hydrolysis (ΔGᵻ) given by

\[
\Delta Gᵻ = RT \ln \left( \frac{\Gamma}{K_{eq}} \right) \quad (2)
\]

where R = 1.987 cal·degree⁻¹·mol⁻¹, T = 310°K, Γ is the mass-action ratio for ATP hydrolysis, and Kₑq represents an equilibrium constant of ~2.19 × 10⁻³ (23).

Malate and pyruvate titration studies were completed at each of three ΔGᵻ, established by appropriate additions of PCr and Cr to the respiration medium. In this case, KCl and distilled, deionized water were manipulated to achieve consistent ionic strength. After a 5-min preincubation period, a 1.0-ml volume of the experimental medium was aspirated from the respiration chamber, quickly added to 100 µl of 10% sodium dodecyl sulfate with rapid mixing, and centrifuged at 14,000 g for 10 min. The resulting supernatant was stored at −80°C for subsequent determination of metabolite concentrations to verify the success of the energy clamp.

The remaining 3.0 ml of respiration medium were used for the pyruvate and malate titration studies. In the malate titration studies, mitochondria (~0.15 mg) were incubated in the presence of saturating (1 mM) pyruvate. Stepwise additions of malate were made to achieve the following concentrations (in mM): 10, 25, 50, 100, 400, and 1,000. Steady-state aerobic energy turnover, as evidenced by a linear trace of oxygen consumption, was established at each malate concentration.

Pyruvate titration studies were similar, but in this case the CAC was primed with saturating (1 mM) malate. Stepwise additions of pyruvate were made to achieve the following concentrations (in mM): 10, 25, 50, 100, 500, and 1,000. Steady-state Jₜ was also established at each of the pyruvate concentrations.

Apparent Kₘ and Vₑₐₙₚ values for both malate and pyruvate were determined from Eadie-Hofstee analyses of Jₜ[S] vs. Jₜ at each energy state, where [S] is the substrate concentration. In addition, apparent Kₘ values for malate and pyruvate were determined under saturating (0.67 mM) ADP conditions (state 3). The same data were also used to evaluate the influence of substrate concentration on the slope of the force-flow (ΔGᵻ–Jₜ) relationship.

Experimental model notes. The concepts of “top-down” MCA (7) can be used to show that the CK energy clamp is a useful experimental model to evaluate the response of mitochondria to changes in substrate availability. With top-down MCA we can conceptually view the aerobic ATP turnover in our in vitro experimental system as two “blocks,” an ATP-generating block (mitochondria) and an ATP-utilizing block (ATPase) such that

\[
\text{fuel} \rightarrow \text{ATP} \rightarrow \text{work} \quad (3)
\]

where the first arrow represents the mitochondrial block, the second arrow represents the ATPase block, and ATP is the common intermediate. In reality, the coupling of the fuel → ATP transduction is imperfect because of operation of the proton leak, but for simplicity, this is neglected in the present discussion because mitochondria subjected to significant flux demand, as in the present experimental system, operate at forces and flows at which the leak flux is relatively small (6). Furthermore, the following discussion treats the ATP shown in Eq. 3 as ATP free energy (ΔGᵻ). The summation theorem of MCA dictates that the sum of the flux control coefficients for the pathway must equal 1.0

\[
C_{\text{mito}} + C_{\text{ATPase}} = 1.0 \quad (4)
\]

where J is the steady-state rate of ATP turnover and C is the flux control exerted by a given block. The elasticity (ε) of a block to the common intermediate describes the response of that block to a change in the level of the intermediate. For example, a fall in ΔGᵻ tends to increase mitochondrial ATP production rate but may exert a braking influence on ATP utilization rate by the ATPase. High elasticity numbers large changes in flux through a block for a given change in intermediate level. In this two-block system, the connectivity property of MCA states that the sum of the flux control coefficients times their respective elasticities to the common intermediate must equal zero.
Accordingly, the ratio of the flux control coefficients must equal the negative reciprocal of the elasticities to the common intermediate

\[ \frac{C^j_{\text{mito}}}{C^j_{\text{ATPase}}} = - \frac{\epsilon^j_{\text{ATPase}}}{\epsilon^j_{\text{sub}}}. \]

(6)

In the CK clamp, the ATPase is CK, which is added in excess to ensure maintenance of near equilibrium, i.e., nearly infinite elasticity (30). Inspection of Eq. 6 reveals that the value of the ATPase flux control coefficient, \( C^j_{\text{ATPase}} \), must therefore be nearly zero, because the flux control coefficients must sum to unity (Eq. 4) and \( \epsilon^j_{\text{ATPase}} \sim \infty \). Thus, in our experimental system, the mitochondrial block contains essentially all of the flux control; \( C^j_{\text{mito}} \sim 1.0 \). The dependence of mitochondria on fuel availability was examined by using another MCA concept, the partitioned response coefficient. In this two-block model

\[ R^j_{\text{subrate}} = C^j_{\text{mito}} \cdot \epsilon^j_{\text{sub}} + C^j_{\text{ATPase}} \cdot \epsilon^j_{\text{ATPase}} \]

(7)

which states that system flux will respond to an external effector, in this case added substrate, depending on the elasticities of the blocks to the effector times the respective flux control coefficient of the block. In the present experiments, oxidative substrate level, pyruvate or malate, was added to influence metabolic flux. Because the mitochondrial block contains all flux control (\( C^j_{\text{mito}} \sim 1.0 \)), the \( J_o \) response of the system represents the elasticity of the mitochondrial block to fuel availability. In other words, the experimental results reflect the extent to which, and over what concentration range, fuel availability limits mitochondrial flux. Furthermore, the analysis was carried out at three extramitochondrial energy states, which roughly span the range of \( \Delta G_p \) observed in skeletal muscle contracting under steady-state aerobic conditions (28).

Metabolic assays. ATP and ADP were assayed by using enzyme reactions linked to the oxidation or reduction of NAD(P)/NAD(P)+H (3). PCr and Cr were assayed according to the methods described by De Strooper and Marechal (17).

Statistics. Significant differences were identified with repeated-measures analysis of variance. A Tukey honestly significant difference post hoc test was used to indicate differences between specific mean values. Alpha was set at \( P < 0.05 \).

Least squares regression lines were determined for the \( \Delta G_p \)-\( J_o \) relationship at each of the specified pyruvate and malate concentrations. Differences in slopes were analyzed using analysis of covariance (ANCOVA). Again, alpha was set at \( P < 0.05 \). If an ANCOVA indicated a statistically significant difference, post hoc analysis employing the Newman-Keuls multiple range test was used to compare the slopes of the regression lines corresponding to the specified malate and pyruvate concentrations.

RESULTS

Functional integrity of isolated mitochondria. Mitochondrial yield was 1.04 ± 0.05 mg/g wet muscle. Mitochondrial preparations demonstrated excellent functional integrity with \( P + M \) state 3, state 4, RCR, and ADP/O equal to 634.9 ± 22.2 nmol \( O_2 \cdot \text{mg}^{-1} \cdot \text{min}^{-1} \), 68.3 ± 10.90 nmol \( O_2 \cdot \text{mg}^{-1} \cdot \text{min}^{-1} \), 10.5 ± 1.3, and 2.91 + 0.09, respectively (n = 10).

Kinetic analysis of malate oxidation. As reported above, when pyruvate, malate, and ADP were provided at saturating levels (1 mM, 1 mM, and 0.67 mM, respectively) the observed mitochondrial \( J_o \) was 635 nmol \( O_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \) (Table 1). However, in the absence of added malate, mitochondrial \( J_o \) was only 13.3 nmol \( O_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \), or 2.1% of the state 3 rate. We take these data to indicate that isolated muscle mitochondria contain little or no CACI carbon. It follows that the size of the CACI carbon pool available to the respiring mitochondria was very nearly equivalent to the experimentally controlled malate additions.

Our strategy was therefore to provide saturating (1 mM) pyruvate and then to make stepwise additions of malate to determine the apparent \( K_m \) and \( V_{\text{max}} \) for malate, i.e., CACI (Table 1). Furthermore, this procedure was carried out at each of three fixed energy states (\( \Delta G_p \)), −14.7, −13.7, and −13.2 kcal/mol, and in the presence of saturating ADP. The results, shown in Fig. 1A, indicate that under each of the energetic conditions, the elasticity of mitochondrial \( J_o \) to malate appeared to conform to Michaelis-Menten kinetics. Eadie-Hofstee plots, shown in Fig. 1B, yielded the apparent \( K_m \) and \( V_{\text{max}} \) values for malate reported in Table 1. The apparent \( K_m \) for malate oxidation increased as energy state fell. Similarly, the apparent \( V_{\text{max}} \) for malate oxidation rose as energy state declined, reflecting the elasticity of the mitochondrial block to both the thermodynamic and kinetic (thermokinetic) influence of the changing \( \Delta G_p \), ATP/ADP ratio, and free [ADP].

Kinetic analysis of pyruvate oxidation. Similarly, the apparent \( K_m \) and \( V_{\text{max}} \) for pyruvate oxidation were determined at the four energetic conditions. In the absence of a pyruvate addition, isolated mitochondria exposed to saturating ADP and 1.0 mM malate respired at a \( J_o \) of 8.0 nmol \( O_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \), or 1.3% of the state 3 \( P + M \) \( J_o \) reported in Table 2. As with malate above, we take these data to indicate that our isolated skeletal muscle mitochondria carried no significant source of acetyl-CoA, and thus fuel (pyruvate) availability was experimentally established. In the presence of a 1 mM malate CAC priming bolus, pyruvate was added stepwise as oxidative substrate. The resulting elasticities are shown in Fig. 2A. Eadie-Hofstee plots of these data, shown in Fig. 2B, are once again consistent with Michaelis-Menten kinetics. Again, both \( K_m \) and \( V_{\text{max}} \) for pyruvate oxidation rose progressively as energy state declined (Table 2).

Thermodynamic analysis of malate oxidation. The same data set could also be used to evaluate the impact of substrate availability on the relationship between \( J_o \) and \( \Delta G_p \). Previous research from several laboratories indicates a near-linear or quasi-linear relationship between \( J_o \) and \( \Delta G_p \) and, therefore, supports the concept that thermodynamic factors contribute to the control of respiration in vivo (10, 18, 26, 39) and in vitro (16, 49). Consistent with the work of Combs et al. (8), the data shown in Fig. 3 were best described by a quadratic force-flow relationship with an \( R^2 \) value essentially equal to 1.0. A linear fit was also satisfactory, yielding \( R^2 > 0.9 \) at every malate

<table>
<thead>
<tr>
<th>( \Delta G_p ), cal/mol</th>
<th>Apparent ( K_m ) (CACI), ( \mu )M</th>
<th>( V_{\text{max}} ), nmol ( O_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>−14.657</td>
<td>21.2 ± 3.6</td>
<td>61.9 ± 6.7</td>
</tr>
<tr>
<td>−13.666</td>
<td>26.1 ± 4.6</td>
<td>154.2 ± 15.9</td>
</tr>
<tr>
<td>−13.239</td>
<td>32.1 ± 7.1</td>
<td>234.7 ± 17.4</td>
</tr>
<tr>
<td>State 3</td>
<td>61.7 ± 0.1</td>
<td>634.9 ± 22.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. \( \Delta G_p \), the Gibbs free energy of ATP hydrolysis, was established with a creatine kinase energy clamp, and in the presence of saturating (1 mM) pyruvate, malate was added stepwise to give concentrations from 10 mM to 1 mM. \( K_m \) and \( V_{\text{max}} \) were determined from Eadie-Hofstee plots of \( J_o \) vs. \( J_o \), where \( J_o \) is mitochondrial \( O_2 \) consumption rate and [S] is substrate concentration. At each level of \( \Delta G_p \), \( n = 5 \) mitochondrial preparations, respectively. CACI, citric acid cycle intermediate.
concentration. The slope (in units of nmol O$_2$/mol ATP·mg$^{-1}$·min$^{-1}$·kcal$^{-1}$) of the linear fit increased as malate-supported CACI concentrations increased from 10 to 1 mM, ranging from 29.8 (at 10 mM malate) to 106.4 (at 1 mM malate), a 3.6-fold range across the CACI concentration examined (Table 3). The slope increased significantly with each malate increment from 10 to 400 mM. By contrast, increasing malate concentration from 400 to 1,000 mM failed to further increase the slope.

Thermodynamic analysis of pyruvate oxidation. Similarly, the relationship between $J_o$ and $\Delta G_p$ for pyruvate concentrations ranging from 10 μM to 1 mM is illustrated in Fig. 4. Again, quadratic relationships provided a better fit of the data, but linear fits gave slopes that varied by ~4.5-fold across the 10–1,000 μM range of pyruvate concentrations (Table 3). With pyruvate, all force-flow slopes were significantly different from each other.

**DISCUSSION**

**Kinetic analysis of CACI and pyruvate concentrations.** In the terminology of top-down MCA (7), we treated the entire

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**Table 2. Kinetic analysis of pyruvate oxidation**

<table>
<thead>
<tr>
<th>$\Delta G_p$, cal/mol</th>
<th>Apparent $K_m$ (pyruvate), μM</th>
<th>$V_{max}$, nmol O$_2$/mg$^{-1}$·min$^{-1}$·kcal$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>−14,657</td>
<td>12.1 ± 1.2</td>
<td>92.8 ± 16.6</td>
</tr>
<tr>
<td>−13,666</td>
<td>19.6 ± 2.0</td>
<td>256.2 ± 22.4</td>
</tr>
<tr>
<td>−13,239</td>
<td>25.8 ± 3.1</td>
<td>367.4 ± 25.1</td>
</tr>
<tr>
<td>State 3</td>
<td>55.0 ± 6.6</td>
<td>634.9 ± 22.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. $\Delta G_p$ was established with a creatine kinase energy clamp, and in the presence of saturating (1 mM) malate, pyruvate was added stepwise to give concentrations from 10 μM to 1 mM. $K_m$ and $V_{max}$ were determined from Eadie-Hofstee plots of $J_o$ vs. [S]. At each level of $\Delta G_p$, $n = 5$ mitochondrial preparations. For state 3 $K_m$ and $V_{max}$, $n = 2$ and 10 mitochondrial preparations, respectively.

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**Fig. 1.** Michaelis-Menten (A) and Eadie-Hofstee plots (B) under state 3 conditions and at Gibbs free energy of ATP hydrolysis ($\Delta G_p$) values of −14,657, −13,666, and −13,239 cal/mol in the presence of a fixed energy state and saturating (1 mM) malate concentration. Malate was added stepwise to achieve malate-supported citric acid cycle intermediate (CACI) concentrations of 10, 25, 50, 100, 400, and 1,000 μM. The reported mitochondrial respiration rates ($J_o$) represent the steady-state oxidative flux under a given condition.

**Fig. 2.** Michaelis-Menten (A) and Eadie-Hofstee plots (B) under state 3 conditions and at $\Delta G_p$ values of −14,657, −13,666, and −13,239 cal/mol in the presence of a fixed energy state and saturating (1 mM) malate concentration. Pyruvate was added stepwise to achieve pyruvate concentrations of 10, 25, 50, 100, 500, and 1,000 μM. The reported mitochondrial $J_o$ values represent the steady-state oxidative flux under a given condition.
mitochondrial pathway of P + M oxidation as a block. Substrate concentrations were established at constant levels and were thus parameters of the experimental system. Accordingly, the response of mitochondrial oxidative flux to changes in substrate concentration can be regarded in the terms of a response coefficient (Eq. 7). The experimental results indicated that Michaelis-Menten enzyme kinetics satisfactorily described the mitochondrial response to variations in malate and pyruvate concentrations (Eq. 8)

\[ v = \frac{V_{\text{max}}[S]}{K_m + [S]} \]  

(8)

In the near-linear region of a Michaelis-Menten plot, i.e., when \([S] \ll K_m\), Eq. 8 may be simplified to yield Eq. 9

\[ v = \frac{V_{\text{max}}}{K_m} [S] \]  

(9)

In the linear portion of a Michaelis-Menten plot, therefore, a falling CACI or pyruvate concentration ([S]) in the face of unchanging demand (constant \(v\)), would require an increase in the ratio of \(V_{\text{max}}\) to \(K_m\). Because a fall in energy state does indeed increase \(V_{\text{max}}\) (thermokinetic control of oxidative phosphorylation) and, thus, the ratio \(V_{\text{max}}/K_m\) (Table 1), it appears that a falling CACI or pyruvate concentration may be compensated by a full in energy state to restore metabolic flux to the rate demanded, i.e., the steady-state oxygen consumption (\(\dot{V}_{O_2}\)). This was the conclusion reached by Sahlin et al. (43), with particular emphasis on the limitation imposed by falling CACI concentration.

More generally, Eq. 8 can be transformed to solve for the relative reaction velocity of an enzyme or enzyme pathway (Eq. 10)

\[ \frac{v}{V_{\text{max}}} = \frac{[S]}{K_m + [S]} \]  

(10)

This transformation of Eq. 8 depicts relative reaction rate, \(v/V_{\text{max}}\), as a function of the substrate-dependent parameter \([S]/(K_m + [S])\). This analytical framework can be used to view the kinetic data of the present study in the context of representative in vivo concentrations of CACI and pyruvate, to gain quantitative insight into the adequacy of CACI and pyruvate availability during prolonged, submaximal exercise.

The apparent \(K_m\) for CACI in the present study ranged from 21 to 32 \(\mu\)M with a mean of 26 \(\mu\)M in mitochondria operating at the intermediate energetic forces and flows that are observed in vivo (26, 28). Reported values of CACI concentrations at rest and during exercise are much greater than these \(K_m\) values. During exercise at \(\sim 60\% - 80\%\) maximal \(\dot{V}_{O_2}\) (\(\dot{V}_{O_2\text{max}}\)). CACI concentrations range from 1,200 to 2,200 \(\mu\)M in human skeletal muscle (20, 21, 42, 43, 46), as well as in rodent skeletal muscle (1). Applying a mean \(K_m\) for CACI of 26 \(\mu\)M and an average CACI concentration of 1,700 \(\mu\)M to Eq. 10 suggests that the measured CACI concentration would be catalytically competent to support a rate of CAC flux at exhaustion that is \(\sim 99\%\) of maximal predicted flux, i.e., \(v/V_{\text{max}} = (1,700)/(26 + 1,700)\). The present in vitro data thus suggest little impact of physiological variations in CACI concentration on pyruvate oxidation. Gibala et al. (22) have reached similar conclusions based on their in vivo experiments on resting and working skeletal muscle.

By comparison, the \(K_m\) for pyruvate was \(\sim 12 - 26\) \(\mu\)M with a mean of 19 \(\mu\)M. Pyruvate concentrations during steady-state exercise at \(60\% - 80\%\) \(\dot{V}_{O_2\text{max}}\) range from \(\sim 50\) to 200 \(\mu\)M (1,

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Table 3. Effect of substrate concentration on slope of \(\Delta G_p-J_o\) relationship

<table>
<thead>
<tr>
<th>[Substrate], (\mu)M</th>
<th>(\Delta G_p-J_o), mmol O_2/mol ATP/min (^{-1})mg (^{-1})kcal (^{-1})</th>
<th>Malate</th>
<th>Pyruvate</th>
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<tbody>
<tr>
<td>10</td>
<td>29.8±9.5</td>
<td>38.6±7.1</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>53.4±8.2</td>
<td>83.1±3.6</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>77.1±9.2</td>
<td>129.0±4.8</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>95.3±7.9</td>
<td>156.6±8.3</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>107.5±5.0</td>
<td>169.2±9.9</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>106.4±5.2</td>
<td>174.1±13.0</td>
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<td>1,000</td>
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</table>

Values are means ± SE. Slopes of force-flow (\(\Delta G_p-J_o\)) relationships are derived from linear regression analysis of Figs. 3 and 4 for malate and pyruvate, respectively.
Applying a mean \(K_m\) for pyruvate of 19 \(\mu M\) and an average pyruvate concentration of 125 \(\mu M\) to Eq. 2 predicts catalytic competence to support \(87\%\) of maximal predicted flux. With prolonged glycogen-depleting exercise, in vivo pyruvate concentration is likely to be lower; Sahlin et al. (43) reported a pyruvate concentration of \(50\) \(\mu M\) after 75 min of cycle ergometry at 70\% of \(V_{O_2\max}\). This pyruvate concentration would, according to present analysis, predict 72\% of \(V_{max}\).

This in vitro evaluation of fuel adequacy would appear to inditc mainline pyruvate oxidation as a limiting process when carbohydrate reserves are depleted during prolonged moderate exercise. Moreover, because pyruvate and lactate equilibrate and diffuse throughout the entire body water, these data may help to explain the enormous mobilization of glycogen carbon into the lactate pool that Crowley et al. (14) observed in rats after 20 min of moderate aerobic exercise.

However, the analytical framework presented in Eq. 10 predicts that a reaction or pathway invariably operates at some level of substrate deficit. Furthermore, this approach does not provide quantitative insight into the magnitude of the adjustment in energy state required to compensate for a decline in fuel availability.

**Thermodynamic analysis of CACI and pyruvate concentrations.** Comparing the \(\Delta G_p-J_o\) relationship for malate and pyruvate oxidation lends additional insight to an evaluation of substrate adequacy. In Fig. 3, the slope of the \(\Delta G_p-J_o\) relationship rises with increasing CACI concentration. However, increasing CACI concentration from 400 to 1,000 \(\mu M\) failed to further increase the slope. This failure is noteworthy because 1) in our experiments, malate-supported CACI addition accounted for essentially all of the carbon in the citrate cycle, and 2) reported values for in vivo malate concentration alone (neglecting all other CACI) at rest, submaximal exercise, and exhaustion (1, 20, 21, 42, 43, 46) clearly exceed 400 \(\mu M\). Therefore, in vitro at least, decreases in malate concentration or, more generally, malate-supported CACI concentrations in the physiological range, do not require a compensatory decline in \(\Delta G_p\) to sustain a given \(J_o\).

By contrast, a physiological range of pyruvate concentrations (1, 20, 21, 43, 44, 46) including rest, submaximal exercise, and exhaustion is associated with an \(\sim 4.5\)-fold range in the \(\Delta G_p-J_o\) slope. This range of pyruvate concentrations, \(\sim 10–200 \mu M\), is illustrated in Fig. 4. Again, on the basis of these in vitro data, the decrease in pyruvate concentration characteristic of prolonged submaximal exercise would appear to require an obligatory fall in \(\Delta G_p\) to sustain a given \(J_o\). In summary, this thermodynamic evaluation of CACI and pyruvate adequacy is entirely consistent with the preceding kinetic analysis and, thus, provides further support for the concept that pyruvate oxidation may be the primary limiting process during moderate, prolonged aerobic exercise.

**Experimental model.** The purpose of these studies was to provide thermokinetic parameters for malate and pyruvate oxidation under controlled conditions. We believe that such data are essential elements in any attempt to quantitatively evaluate the adequacy of an oxidative fuel and that isolated skeletal muscle mitochondria provide a useful experimental model. Isolated mitochondria are capable of carrying out oxidative phosphorylation and maintain excellent functional integrity, as evidenced by high maximum \(J_o\) and the maintenance of a range of extramitochondrial \(\Delta G_p\) observed in vivo (28, 34). In addition, isolated skeletal muscle mitochondria lack pyruvate and CACI; mitochondrial respiration may therefore be studied at experimentally fixed pyruvate and CACI concentrations and at various steady-state chemiosmotic forces and flows. Nevertheless, it is also important to remember that these data were obtained from experiments conducted in vitro.

In vivo pyruvate flux can be increased through covalent activation of pyruvate dehydrogenase (PDH) (47). In the transition from rest to moderately heavy exercise, PDH activation state is increased by less than fivefold (11). However, resting muscle may derive \(\sim 20\%\) of oxidative fuel from carbohydrate (15, 40, 48), whereas under the exercising conditions above, skeletal muscle derives \(>90\%\) of its fuel from carbohydrate (41). Thus, if we assume that the rate of exercising \(O_2\) consumption exceeds rest by, for example, roughly 50-fold, then it appears that the rate of pyruvate oxidation increases, according to this estimation, by 250-fold from rest to exercise. We therefore interpret the present results showing dependence of mitochondrial oxidative flux on pyruvate concentration alterations in the physiological range as a mechanism that acts in concert with PDH activation to effect the large flux changes observed in the transition from rest to exercise in skeletal muscle.

Pyruvate can activate pyruvate dehydrogenase complex (PDC) through its inhibitory effect on pyruvate dehydrogenase kinase (PDK), the enzyme that functions to phosphorylate and inactivate PDC. Thus the argument might be advanced that the results of the present studies merely reflect the impact of pyruvate availability on PDC activation state, rather than on pyruvate concentration per se. We tested this possibility by performing parallel experiments using 5 mM dichloroacetate (DCA), which ensured full activation of PDC (51). Results of these experiments (data not shown) demonstrated no effect of DCA on the kinetic parameters or \(\Delta G_p-J_o\) relationships. These results were consistent across all three energy states evaluated in the initial studies. Thus the observed effect of pyruvate concentration on \(V_{max}\) and the \(\Delta G_p-J_o\) slope was independent of PDC activation state.

In summary, the present study provides quantitative evaluation of the adequacy of CACI and pyruvate in the support of oxidative energy production in isolated skeletal muscle mitochondria. \(K_m\) and \(V_{max}\) for both pyruvate- and malate-supported CACI oxidation rose in response to decrements in energy state. Corresponding thermodynamic analysis indicated an essentially constant slope of the \(\Delta G_p-J_o\) relationship across a physiological range of malate-supported CACI concentrations. By comparison, an approximate fourfold variation in the slope of the \(\Delta G_p-J_o\) relationship was observed across a physiological range of pyruvate concentrations. Notwithstanding the role of pyruvate concentration in modulating the activation state of PDC, the effect of pyruvate concentration on this fourfold variation in the slope of the \(\Delta G_p-J_o\) relationship was shown to be independent of PDC activation state.

These kinetic and thermodynamic relationships, obtained under in vitro conditions, may thus be interpreted to suggest that the progressive declines in pyruvate availability characteristic of prolonged, submaximal exercise appear to require compensatory declines in \(\Delta G_p\) to sustain a given rate of oxidative energy production. Accordingly, we concur with the views of Gibala et al. (22) that the role of pyruvate in mainline...
 oxidation appears to represent the more promising mechanism for future studies of the association between CHO depletion and fatigue during prolonged, submaximal exercise.

**Perspectives**

The central nervous system (CNS) stimulates energy turnover in skeletal muscle by activating the ATPase block as shown by Eq. 3. A recent MCA analysis of working human skeletal muscle by Jeneson et al. (27) has shown that ATPase activity dominates flux control at low energy turnover but that control is redistributed toward the mitochondrion as flux rises (and energy state falls) into the moderate aerobic range. The present results indicate that mitochondrial elasticity to $\Delta G_P$ falls with decreases in pyruvate concentration. According to Eq. 6, this implies that falling pyruvate concentration results in a redistribution of control toward the mitochondrion and away from the ATP-utilizing sites. The implication is that the CNS loses control of muscle energy turnover as carbohydrate fuel becomes exhausted. This conclusion is consistent with the observation that as muscle glycogen and pyruvate levels fall, additional motor units are recruited to sustain moderate aerobic exercise (45). Our laboratory previously proposed a similar mechanism to explain why iron-deficient rats subjected to very mild exercise training (slow walking on a level treadmill) demonstrate marked mitochondrial adaptations in fast white skeletal muscle (52). Those experiments showed that dietary iron deficiency profoundly altered the elasticity of skeletal muscle mitochondria to $\Delta G_P$. The work of Hogan, Arthur, and colleagues (2, 25), manipulated $O_2$ as a limiting substrate of oxidative phosphorylation to show the connectivity property in canine muscle in situ. Implicit in this view is the hypothesis that the contractile apparatus has a finite (negative) elasticity to $\Delta G_P$, or metabolites linked to it via the creatine kinase and adenylate kinase equilibria (9). Indeed, the contractile function of mammalian striated muscle is impaired by rising [Pi] (12, 19, 20). GP, or metabolites linked to it via the creatine kinase model.

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