1993

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Recommended Citation
Published in Comparative Biochemistry and Physiology.,106B: 889-893, 1993

AN ANALYSIS OF pH TOLERANCE AND SUBSTRATE PREFERENCE OF ISOLATED SKELETAL MUSCLE MITOCHONDRIA FROM *BUFO MARINUS* AND *RANA CATESBEIANA*

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Abstract—1. The effects of varying pH and substrate on isolated skeletal muscle mitochondria from *Bufo marinus* and *Rana catesbeiana* were investigated.
2. For both species, $\dot{V}O_2$ max significantly decreased at all pH < 7.3 ($P < 0.05$), while maximum values were observed at a pH range of 7.3–7.6 with *B. marinus* maintaining a greater $\dot{V}O_2$ max than *R. catesbeiana*.
3. Respiratory control values (RCR) decreased significantly at all pH < 6.9 for both species ($P < 0.05$).
4. Isolated mitochondria from both species were maintained at pH = 7.2 and O_2 consumption measured under five separate substrate conditions.
5. A rank preference was established based upon state 3 and RCR values.
6. Substrate preference was identical for both species and interspecific comparisons revealed differences in state 3 respiration and coupling.

INTRODUCTION

There is a limit to the rate of oxygen consumption ($\dot{V}O_2$ max). Two predominating hypotheses exist to explain this limitation. The first hypothesis, that the number of skeletal muscle mitochondria is rate-limiting in itself, is supported by studies evaluating citrate synthase activity (Putnam and Bennett, 1983; Cummings, 1979), and correlations of $\dot{V}O_2$ max with number or density of mitochondria (Hoppeler, 1990; Hoppeler *et al.*, 1987). The second hypothesis contends that $\dot{V}O_2$ max is governed by systemic oxygen transport (see Hillman *et al.*, 1985).

Both hypotheses, however, consider only oxygen influx and not the possible role of carbon dioxide efflux. Uptake of oxygen by mitochondria dictates the production of carbon dioxide. If carbon dioxide is not effectively removed at the cellular level, the resulting decrease in intracellular pH may have a detrimental effect on mitochondrial function and hence $\dot{V}O_2$ max. This investigation evaluates mitochondrial performance at five pH values in order to determine the effect of decreasing pH on mitochondrial function.

Substrate utilization by mitochondria *in vitro* reflects the possible array of molecules oxidized *in vivo*. Studies on the oxidative properties of carp muscle (Moyes *et al.*, 1989; Johnston, 1977) suggest that mitochondria from different fiber types (i.e. red or type I, and white or type II) are specialized for oxidation of different sets of substrates as reflected by their substrate preference. To date, little data is available for amphibian mitochondrial substrate preference. Existing studies on substrate oxidation by frog skeletal muscle mitochondria include data for pyruvate/malate, succinate, and several acylcarnitines of varying lengths (Skoog *et al.*, 1978; Osmundson and Bremer, 1978). However, no individual study addresses substrate preference over a wide range of fuels.

The following study delineates a mitochondrial pH profile for both *Bufo marinus* and *Rana catesbeiana*. Isolated skeletal muscle mitochondria performance is evaluated over a pH range of 6.1–7.6. This range encompasses normal physiological extremes based on blood pH measured from amphibians at rest and following exhaustive exercise (Putnam, 1979), and assuming a blood–cell ΔpH of −0.4 (see Roos and Boron, 1983). If mitochondrial oxygen consumption diminishes with decreasing pH, then the possibility of carbon dioxide efflux directly affecting oxygen metabolism is possible. In addition, substrate preference of isolated skeletal muscle mitochondria from both the toad and frog was assayed and compared.

MATERIALS AND METHODS

Animals

Bufo marinus were purchased from a commercial supplier while *Rana catesbeiana* were captured from local wild populations in Washington County, OR. Both species were maintained in the laboratory and

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given free access to water. Experiments were performed within 3 weeks of arrival for toads and 2 weeks of capture for frogs.

Isolation of mitochondria

Animals were doubly pithed and the thigh musculature quickly excised with ice-cold scissors. Approximately 5 g of muscle was weighed in a beaker containing an isolation medium consisting of 170 mM mannitol, 55 mM sucrose, 5 mM EGTA (ethyleneglycol-bis-β-aminoethyl ether), 20 mM Tris (2-amino-2-hydroxymethyl-1,3-propanediol), 0.5% bovine serum albumin (BSA) and 50 units/ml of heparin. The final osmotic pressure of 270 mOsm was verified with a vapor pressure osmometer (Wescor Inc. Model 523A). Muscle tissue was manually minced with scissors and placed in a bladed homogenizer for approximately 30 sec at 1000 rpm. The homogenate was forced by a threaded piston through 0.8 mm diameter holes in a custom fabricated tissue press, placed into a Potter-type glass homogenizer (Tri-R Instruments) and ground with the first two (toad) or all three (frog) pestles with clearances of: 0.15, 0.05 and 0.025 mm, at 200 rpm.

The resulting homogenate was spun at 755 *g* for 5 min at 5 C to separate large cellular debris from the mitochondria, which remained in the supernatant. The preserved supernatant was spun at 9800 *g* for 9 min at 5 C to pellet mitochondria. The pellet from the second spin was resuspended in isolation medium and centrifuged a third time (to yield a more pure mitochondrial isolation) for 7 min at 9800 *g* and 5°C. The final resuspension involved 200 μl of isolation medium with BSA being gently mixed into the second pellet with a glass stirring rod forming a mitochondrial slurry. A 50 μl (toad) or 20 μl (frog) aliquot was frozen for protein determination.

Measurement of respiration

Oxygen uptake was measured at 20°C with a Transidyne General Chemical Microsensor Model 1251 calibration cell. Attached to the 0.9 ml reaction chamber was an oxygen microelectrode, which utilized the polarographic principle to measure oxygen tension within the chamber (see Estabrook, 1967). The output voltage of the system was recorded on a strip-chart recorder.

For all assays, the reaction chamber was filled with 870 μl of an oxygen uptake medium which consisted of 55 mM mannitol, 24 mM sucrose, 10 mM Tris (pH = 7.2 at room temperature), 10 mM KH_2PO_4, 90 mM KCl, and 50 mM malic acid (osmotic pressure = 283 mOsm). A high concentration of malate insured against transporter limitation, and since rank order comparisons are made, the endogenous rate of malate oxidation is cancelled. In combination with oxygen uptake media, 10 μl of mitochondrial suspension and saturating concentrations of substrate were present. Maximal oxidative activity was initiated with the addition of 0.5 mM ADP.

For assays involving pH effects, the pH of the oxygen uptake medium was adjusted to 6.1, 6.5, 6.9, 7.3, or 7.6 at room temperature with either HCl or NaOH. Pyruvate (11 mM) is a substrate with known high oxidation rates in amphibian skeletal muscle mitochondria (Hillman *et al.*, 1991). Under saturating conditions, pyruvate transport is not carrier-mediated or pH-dependent in the range studied here (Pande and Parvin, 1978); consequently, pH does not affect substrate transport. For assays involving substrate preference, the pH of the oxygen uptake medium was held constant at 7.2. Although a pH optimum of >7.3 was determined with data from the first half of this experiment, a pH of 7.2 was believed to be an appropriate simulation of *in vivo* conditions (see Roos and Boron, 1983) during steady-state respiration. Each substrate was added to separate assay chambers to a concentration of 11 mM, except octanoylcarnitine, which was added to 2.2 mM. All biochemicals were obtained from Sigma Chemical Co. (St Louis, MO).

Upon the addition of 0.5 mM ADP, mitochondrial oxidative phosphorylation was measured as the difference in O_2 tension within the reaction chamber per minute. This rate of oxygen consumption is considered either state 3 or $\dot{V}O_2$ max (under saturating conditions). Respiratory control ratios (RCR) were calculated as the ratio of the rate of oxygen uptake in the presence of ADP (state 3) to the rate of oxygen uptake after exhaustion of ADP (state 4) or state 3/state 4 (Estabrook, 1967; Chance and Williams, 1956). Aliquots of mitochondrial suspensions were thawed, diluted and added to Folin phenol reagent. Concentration of mitochondrial protein was based on absorbance at 625 nm after Lowry *et al.* (1951). Final oxygen consumption was expressed as nm O_2 consumed per mg mitochondrial protein per minute.

All statistical comparisons were made with ANOVA, using Scheffe's tests for multiple comparisons, unless specifically noted. A $P < 0.05$ was considered significant. Interspecific comparisons between the toad and frog were performed using independent Student's *t*-tests.

RESULTS

For both *B. marinus* and *R. catesbeiana* state 3 oxygen consumption rates declined significantly at all pH < 7.3 ($P < 0.05$) and state 4 values were significantly different at H = 6.1 ($P < 0.05$), Respiratory control ratios for *B. marinus* decreased significantly at all pH < 6.9, and for *R. catesbeiana* all RCR values were significantly different from each other in the assayed pH range ($P < 0.05$), (Figs 1 and 2). At pH = 6.1, all state 4 values were indistinguishable from state 3 (i.e. uncoupled) and consequently RCR values were considered equal to one for *B. marinus* and for *R. catesbeiana*, none of the three respiratory measurements were observed at pH = 6.1.

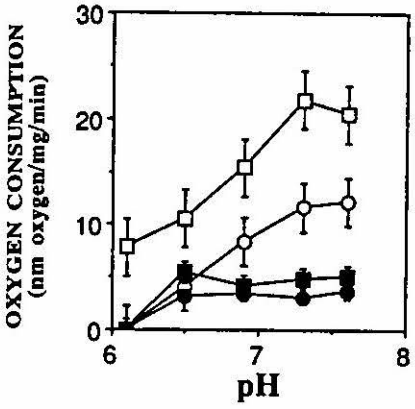

Fig. 1. The effect of pH on state 3 respiration and state 4 respiration in isolated skeletal muscle mitochondria from both anuran species. Values are mean ± standard error. Squares = *Bufo marinus* and circles = *Rana catesbeiana*. Unfilled symbols = state 3 and filled symbols = state 4.

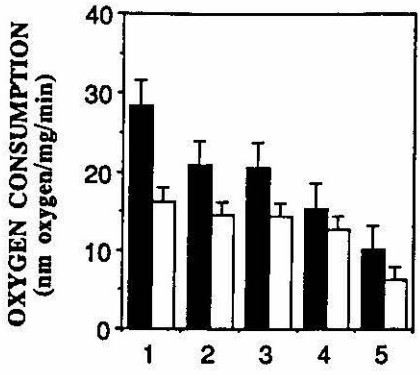

Fig. 3. State 3 substrate preference in isolated skeletal muscle mitochondria. Column 1 = alpha-ketoglutarate, 2 = pyruvate, 3 = octanoyl, 4 = beta-hydroxybutyrate, 5 = oxaloacetate. Values and mean ± standard error. Filled bars = *Bufo marinus* and unfilled bars = *Rana catesbeiana*.

State 3 respiratory rates for *B. marinus* of the five individual substrates all showed significant differences ($P < 0.05$) from each other with the exception of pyruvate vs octanoyl. Preference of substrate in order of greatest to least is as follows: alpha-ketoglutarate, pyruvate, octanoyl–carnitine, beta-hydroxybutyrate and oxaloacetate (Fig. 3). There were no significant differences among the state 4 substrate oxidation measures (Fig. 4). All RCR values for substrate oxidation were significantly different from each other. Substrate preference ranked by RCR values matched that set by state 3 values (Fig. 5).

State 3 values for *R. catesbeiana* were all significantly different with the exception of pyruvate vs octanoyl. The preferred substrate ranking is identical to *B. marinus*, (Fig. 3). State 4 data from *R. catesbeiana* showed significant differences with the following four exceptions: pyruvate vs octanoyl, pyruvate vs beta-hydroxybutyrate, alpha-ketoglutarate vs oxaloacetate, and octanoyl vs beta-hydroxybutyrate (Fig. 4). Respiratory control ratios were all significantly different from each other in *R. catesbeiana*. Substrate preference as determined using RCR values presented the following rank order: octanoyl-carnitine, alpha-ketoglutarate, pyruvate, beta-hydroxybutyrate and oxaloacetate (Fig. 5).

Interspecific statistical comparisons made with the five substrates using state 3 data showed that *B. marinus* had a significantly higher $\dot{V}O_2$ ($P < 0.05$) in four of the five substrates. Oxaloacetate was the only substrate that showed no significant difference in utilization between the two species. RCR data revealed no significant differences with any of the substrates, confirming consistent quality of recovery and good mitochondrial membrane integrity.

DISCUSSION

During intense metabolism, accumulation of both CO_2 (aerobically), and lactate (anaerobically) are unavoidable and result in unfavorable acidic con-

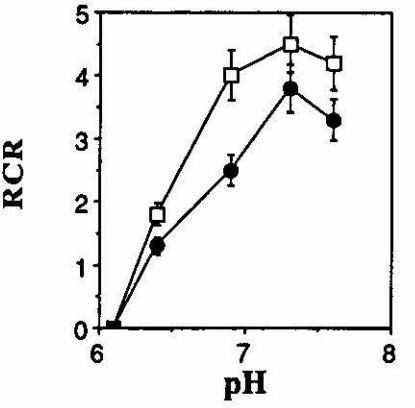

Fig. 2. The effect of pH on respiratory control ratios in isolated skeletal muscle mitochondria from *Bufo marinus* and *Rana catesbeiana*. Values are mean ± standard error. Squares = *Bufo marinus* and circles = *Rana catesbeiana*.

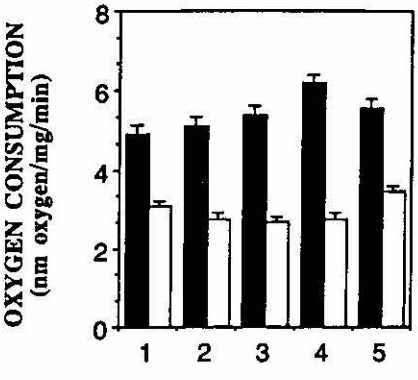

Fig. 4. State 4 substrate preference of isolated skeletal muscle mitochondria. Column 1 = alpha-ketoglutarate, 2 = pyruvate, 3 = octanoyl, 4 = beta-hydroxybutyrate, 5 = oxaloacetate. Values and mean ± standard error. Symbols same as Fig. 3.

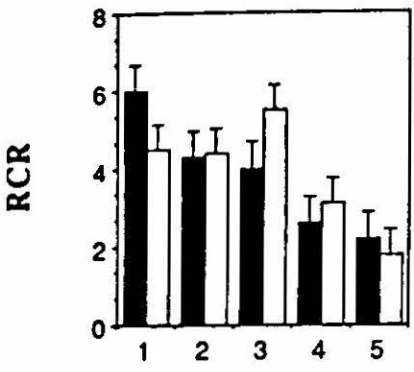

Fig. 5. RCR substrate preference of isolated skeletal muscle mitochondria. Column 1 = alpha-ketoglutarate, 2 = pyruvate, 3 = octanoyl, 4 = beta-hydroxybutyrate, 5 = oxaloacetate. Values and mean ± standard error. Symbols same as Fig. 3.

ditions. Putnam (1979) reported muscle fatigue associated with lactate accumulation and lowered pH during anaerobic exercise.

The data support a pH optimum. A decrease in intracellular pH, possibly as the result of an inadequate CO_2 efflux *in vivo*, may be detrimental to mitochondrial activity and ultimately limit $\dot{V}O_2$ max. Differences in pH may affect mitochondrial carrier protein performance through altered oxidation states of certain amino acids, and/or disturbed tertiary quaternary structure of proteins. Proton binding sites on carrier proteins govern certain transport processes (LaNoue and Schoolwerth, 1979).

The preparation demonstrated that maximum $\dot{V}O_2$ is a direct function of extramitochondrial pH (pH_e). This point is illustrated in Fig. 1. Decreasing pH_e leads to a corresponding decrease in respiration. These results corroborate existing pH studies on invertebrate mitochondria in which a similar relationship between pH and $\dot{V}O_2$ is evident (Ballantyne and Storey, 1983, 1984). Our results are in conflict with Moyes *et al.* (1988), in which increasing state 3 and RCR values were associated with decreasing pH_e values, a result that is not consistent with a chemiosmotic model for oxidative phosphorylation in mitochondria.

Both species preferred each of the five substrates in identical order, indicating multidimensional aerobic capability for each. However, mitochondrial $\dot{V}O_2$ max in *B. marinus* was significantly greater than that of *R. catesbeiana* in all instances except when oxaloacetate was given as a substrate. In contrast, substrate preference in carp red and white muscle mitochondria (Moyes *et al.*, 1989) demonstrated a profound difference: white muscle remained primarily glycolytic, whereas red muscle relied on a wider range of fuels, especially fatty acids, which would yield a greater number of ATP molecules after complete oxidation. This curious disparity may be explained by the fact that carp have histologically distinct red and white muscle fibers while anuran locomotory muscles are arranged in mixed groups (Putnam and Bennett, 1983; Mendiola *et al.*, 1991).

Generally, pyruvate is oxidized at rates as great as any other substrate in vertebrate muscle (Moyes *et al.*, 1990). However, for anuran amphibians, alpha-ketoglutarate is oxidized at a greater rate. Rates of oxidation for beta-hydroxybutyrate, and oxaloacetate were in agreement with data obtained from other vertebrates (Moyes *et al.*, 1990). In both the frog and the toad, octanoylcarnitine and pyruvate were oxidized at similar rates. These data are compared to Skoog *et al.* (1978) in which frog skeletal muscle mitochondria also oxidized palmitoyl-carnitine and pyruvate at similar rates. This indicates an ability of amphibian thigh muscle mitochondria to utilize C-8 or C-16 fatty acids as an alternate source of substrate. Osmundsen and Bremer (1978) report oxidation of octanoylcarnitine at 80% of palmitoyl-carnitine in frog heart mitochondria. Hence, it appears that a preferential difference between oxidation of C-8 and C-16 acylcarnitines is detectable in heart muscle.

The data support the conclusion that pH balance is important for maximal mitochondrial oxidation, and implicate carbon dioxide efflux as a potential limit to $\dot{V}O_2$ max. Substrate preference data is consistent with that obtained for other vertebrates and supports a mixed muscle fiber type theory for anuran amphibians. It appears that both the frog and toad are adapted for aerobic metabolism as reflected by their identical substrate preference. However, the lower mitochondrial $\dot{V}O_2$ max and lower mitochondrial density of the frog relative to the toad indicates lower aerobic muscle metabolism.

Acknowledgements—The authors wish to thank Dr Malcolm Lea for his technical assistance. This work is part of a thesis submitted in partial fulfilment of the requirements for the Master of Science degree by J. Duerr at Portland State University. The authors also wish to thank committee members Larry Crawshaw, Deborah Duffield and Gordon Kilgour.

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