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Fiber-type-related differences in the enzymes of a proposed substrate cycle

Richard A. Howlett *, Wayne T. Willis

Exercise and Sport Research Institute, Dept. of Exercise Science and Physical Education, Arizona State University, Tempe, AZ, 85287-0404, USA

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Abstract

A substrate cycle between citric acid cycle (CAC) intermediates isocitrate and 2-oxoglutarate, involving NAD^+- and $NADP^+$-linked isocitrate dehydrogenase (NAD-IDH and NADP-IDH, respectively) and mitochondrial transhydrogenase (H^+-Thase), has recently been proposed. This cycle has been hypothesized to enhance mito-chondrial respiratory control by increasing the sensitivity of NAD-IDH to its modulators and allowing for enhanced increases in flux through this step of the CAC during periods of increased ATP demand. The activities of the enzymes comprising the substrate cycle: NAD-IDH, forward and reverse NADP-IDH, and forward and reverse H^+-Thase, along with the activity of a marker of mitochondrial content, citrate synthase (CS) were measured in mitochondria isolated from rabbit Type I and Type IIb muscles and in whole muscle homogenates, representing the various fiber types, from rats. In isolated rabbit muscle mitochondria, NAD-IDH had significantly higher ($1.6\times$) activity in white muscle while forward NADP-IDH, forward and reverse H^+-Thase, and CS all had significantly higher ($1.2–1.6\times$) activities in red muscle. There was no difference in reverse NADP-IDH between fiber types. Similarly, in rat whole muscle enzyme activities normalized to CS, NAD-IDH had significantly higher activity in fast-twitch glycolytic (FG) fibers, while forward NADP-IDH and forward H^+-Thase had significantly higher activities in slow-twitch oxidative (SO) fibers. These results suggest that differences in the activities of the substrate cycle enzymes between skeletal muscle fiber types could contribute to differences in respiratory control due to differential cycling rates and/or loci of control. © 1998 Elsevier Science B.V.

Keywords: Skeletal muscle; Isocitrate dehydrogenase; Mitochondrial transhydrogenase; Respiratory control

1. Introduction

Although the control of citric acid cycle (CAC) flux is most likely shared among a number of enzymes, isocitrate dehydrogenase (IDH) is considered to be one important regulator [1]. This enzyme exists in two distinct forms, NAD^+-linked (NAD-IDH) and $NADP^+$-linked (NADP-IDH) [1,2], both of which are found primarily in the mito-chondrial matrix in skeletal muscle [3]. It is held that most CAC flux occurs through NAD-IDH [1,4], despite the nearly 10-fold higher specific activity of NADP-IDH in whole mixed muscle [3,5,6]. The function of NADP-IDH remains a matter of speculation [6]. The occurrence of two distinct forms of IDH, each requiring different cofactors, along with their close relationship to mitochon-

* Corresponding author. Present address: Dept. of Human Biology and Nutritional Sciences, University of Guelph, Guelph, ON, Canada N1G 2W1. Fax: +1-519-763-5902; E-mail: rhowlett@uoguelph.ca

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drial transhydrogenase (H^+-Thase), points to a possible regulatory role [1,2].

Sazanov and Jackson [6], using enzyme activity data from heart muscle mitochondria, proposed that NAD^+- and $NADP^+$-linked IDH could operate in a substrate cycle between iscocitrate (IC) and 2-oxoglutarate (OG) to fine-tune the regulation of CAC flux [2]. Other substrate cycles that have been identified lend themselves to regulatory roles through amplification of sensitivity [7]. When the cycle's forward reaction is balanced by its opposing back reaction, net flux through the step is low, but smaller changes in the concentrations of modulators can greatly enhance forward flux by simultaneously stimulating the forward reaction and inhibiting the back reaction. In the proposed cycle (Fig. 1), NADH is first produced by the NAD-IDH forward reaction, and this NADH then reduces NADP via the transhydrogenase reaction, producing NAD^+ and NADPH. This $NADP^+$ reduction is known as the forward H^+-Thase reaction. To complete the cycle, the NADPH is then oxidized by NADP-IDH operating in the reverse (OG → IC) direction. Because mitochondrial isocitrate oxidation proceeds with a loss of free energy, and because the redox couples NAD/NADH and NADP/NADPH have similar midpoint potentials [8], operation of the cycle requires dissipation of a driving force, a characteristic of all substrate cycles [9]. In the substrate cycle proposed [2], the driving force for the NADP-IDH back reaction (OG → IC) is the greater level of NADP reduction (i.e., $NADPH/NADP^+$ compared to $NADH/NAD^+$), which comes at the expense of the mitochondrial protonmotive force (Δp) due to the translocation of protons from the extramitochondrial space to the mitochondrial matrix by the mitochondrial transhydrogenase [10]. The stoichiometry of the H^+-Thase is approximately 1 H^+ pumped for each pair of electrons transferred from NADH to NADPH [11,12]. Thus, the energy cost of one turn of the cycle is one proton, about 10% of the energy derived from the oxidation of one NADH by electron transfer [8].

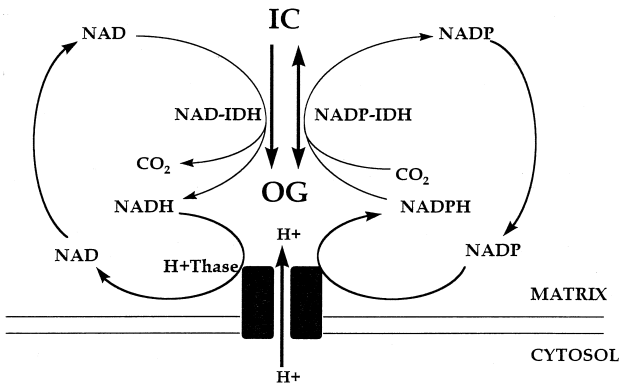

Fig. 1. Representation of the proposed isocitrate-2-oxoglutarate substrate cycle. See text for abbreviations.

Mitochondria isolated from Type IIb skeletal muscle have about two-fold higher NAD-IDH specific activity than mitochondria isolated from Type I muscle [13]. This could contribute to the lower apparent K_mADP for respiration in white muscle shown in both intact tissue [14], and isolated mitochondria [15], especially considering the dependence of NAD-IDH on ADP. Although metabolic differences between red and white muscle result from quantitative differences in cellular mitochondrial content, recent evidence has additionally shown significant compositional differences in important rate-controlling, oxidative enzymes in mitochondria isolated from rabbit white (gracilis) and red (soleus) skeletal muscle [13]. If the proposed substrate cycle exists, it is likely that the mitochondrial enzymes comprising it would show differences in catalytic potential, accounting for some of the differences in metabolic control between red and white fibers.

In order to explore whether the enzymes of the proposed isocitrate- 2-oxoglutarate cycle are kinetically competent to support cycling, this study measured maximal activities of NAD-IDH, NADP-IDH (reverse), and H^+-Thase (forward) in mitochondria isolated from Type I and Type IIb muscle fiber types from rabbits. In addition, activities of forward NADP-IDH and reverse H^+-Thase were evaluated in both isolated rabbit muscle mitochondria as well as whole tissue activities in three fiber types from rats. Citrate synthase activity was also measured in both isolated rabbit muscle mitochondria and whole rat muscle homogenates.

2. Materials and methods

2.1. Animal care

All procedures were in accordance with the guidelines set forth by the Arizona State University Animal Care and Use Committee.

2.2. Tissue preparation

All biochemicals were obtained from Sigma (St. Louis). Mitochondria were prepared from the gracilis (GRAC) and soleus (SOL) of female New Zealand white rabbits ($n = 5$) using the procedures of Makinen and Lee [16]. The entire soleus (98% Type I) and gracilis (99% Type IIb) muscles were excised and placed in ice-cold solution I (100 mM KCl, 40 mM tris(hydroxymethyl) amino-methane (Tris)–HCl, 10 mM Tris–base, 5 mM $MgCl_2$, 1 mM EDTA and 1 mM ATP). Samples were then cleaned of all fat and connective tissue, minced, and treated with protease (Nagarse, 5 mg/g) in 9 vol. of solution I for 7 min. After stopping digestion with an equal volume of solution I, the mixture was then homogenized for 15 s at 40% power with an Ultra-Turrax blender (Cincinnati, OH) blender and centrifuged (Beckman centrifuge model J2-21 M/E) at $600 \times g$ for 10 min. The resulting supernatant was decanted through 4 layers of cheesecloth and centrifuged again for 10 min at $14\,000 \times g$. The remaining pellet was resuspended in solution II (100 mM KCl, 40 mM Tris–HCl, 10 mM Tris–base, 1 mM $MgSO_4$, 0.1 mM EDTA, 0.2 mM ATP, and 1.5% fatty acid-free bovine serum albumin) using a Dounce glass on glass manual tissue grinder, and centrifuged at $7000 \times g$ for another 10 min. This pellet was resuspended in 20 ml of a solution identical to solution II, but without BSA and centrifuged a final time at $3500 \times g$ for 10 min. The final pellet was then suspended in 250–400 μl of buffer containing 200 mM sucrose, 70 mM mannitol, 10 mM Tris–HCl, and 1 mM EDTA (pH 7.4). All buffers and glassware were kept ice-cold throughout the isolation procedures. Mitochondrial suspensions then underwent four cycles of sonication at 40% power for 15 s (Branson sonifier 250) interposed with liquid nitrogen freeze–thaw to prepare them for enzyme assays.

To prepare muscle homogenates (10% w/v) from the rat muscles ($n = 7$), the soleus (SOL), deep vastus (DV), and superficial vastus (SV), (representing primarily SO (87%), FOG (56%), and FG (97%) fibers, respectively [17]) were sampled and prepared for enzyme assays. The muscles were first cleaned of adipose and connective tissues. They were then manually minced in a buffer of 10 mM KH_2PO_4, pH 7.4. Each sample was homogenized with an Ultra-Turrax blender for 30 s at 40% power. Next, samples underwent four cycles of sonication, as above, interposed with freeze–thaw. Finally, samples were centrifuged at $750 \times g$ for 10 min to pellet down contractile proteins and particulate matter, and the supernatant was used for analysis.

2.3. Enzyme assays

All enzymes were assayed spectrophotometrically at 25°C, pH 7.4. Isocitrate dehydrogenase (NAD^+ and $NADP^+$) was assayed according to Chen and Plaut [18]. To assay the forward (IC → OG) IDH reactions, isocitrate (8 mM), 0.7 mM ADP, and either NAD^+ or $NADP^+$ (0.33 mM) were added to 10–100 μl of sample in a buffer containing 33.3 mM Tris acetate, 1.3 mM $MnCl_2$, and 0.05% Triton, and the appearance of NAD(P)H was followed at A_{340} using a millimolar extinction coefficient of 6.23. To assay the backward reaction of NADP-IDH, 0.01 mM NADPH, 0.6 mM OG, 20 mM $NaHCO_3$, and 0.7 mM ADP were added, and the disappearance of NADPH was followed at A_{340} using 6.23 as an extinction coefficient [19]. Transhydrogenase activity was assayed using analogs of NAD^+ and $NADP^+$, 3-acetylpyridine adenine dinucleotide ($AcPyAd^+$) and thio$NADP^+$, respectively [19,20]. Homogenate samples (50–200 μl) were incubated with 2.5 μM rotenone and either 200 μM NADH and 100 μM thio$NADP^+$ (forward reaction) or 200 μM NADPH and 200 μM $AcPyAd^+$ (reverse reaction) [19]. The appearance of the reduced form of the analog was followed at A_{395} (thio$NADP^+$) or A_{375} ($AcPyAd^+$), using millimolar extinction coefficients of 11.3 [12,20] and 6.1 [19], respectively. Note that the measures of transhydrogenase activity are the non-energy linked rates, measured in solubilized mitochondrial protein.

Citrate synthase was assayed as per Srere [21] by incubating sample with 0.5 mM oxaloacetate, 0.1 mM Acetyl–CoA and 0.1 mM DTNB (Ellman's Reagent) in 0.1% Triton in H_2O. The mitochondrial preparations were diluted 1:60, and the 10% homogenates were diluted 1:10 in 0.1% Triton X-100, and 30 μl of sample was used. Appearance of the mercaptide ion was followed at A_{412} using a millimolar extinction coefficient of 13.6.

All enzyme activities were expressed per mil-

Table 1
Enzyme activities of mitochondria isolated from rabbit soleus and gracilis muscle

	NAD-IDH	NADP-IDH (forward)	NADP-IDH (reverse)	Forward H^+-Thase	Reverse H^+-Thase	CS
SOL ($n=5$)	51.79 ± 6.43 *	1694.52 ± 119.79 *	44.42 ± 11.76	8.46 ± 1.00 *	181.43 ± 25.10 *	2193.69 ± 129.56 *
GRAC ($n=5$)	81.52 ± 7.57	1073.93 ± 97.93	45.31 ± 4.32	5.73 ± 0.72	124.41 ± 9.64	1898.00 ± 229.69

Values are means ± SE.
Rates expressed as nmol mg mitochondrial protein^{-1} min^{-1}.
* Significant ($P < 0.05$) difference between soleus and gracilis.

ligram of mitochondrial protein (rabbit), as determined by the method of Lowry et al. [22], or per gram of wet muscle weight (rat).

2.4. Statistical analysis

Differences in specific activities of the rabbit isolated mito-chondrial enzymes were evaluated using independent *t*-tests. To evaluate rat fiber type specific enzyme activity differences, rat whole muscle activities were first normalized to citrate synthase to correct for the effects of mitochondrial content differences between fiber types. Enzyme activities were divided by corresponding CS activities (and multiplied by 100). This normalized rat muscle data was analyzed using a one-way ANOVA, followed by a Student Newman–Keuls test for differences between means.

3. Results

3.1. Rabbit muscle

Mitochondrial specific activities of enzymes from rabbit muscle fiber types are given in Table 1. The specific activity of NAD-IDH was 57% higher ($P < 0.05$) in GRAC than SOL. In contrast, the two enzymes that comprise the 'back reactions' of the proposed IC-OG cycle, forward H^+-Thase and reverse NADP-IDH, exhibited a much different pattern. Forward H^+-Thase was 48% higher in SOL than in GRAC, while reverse NADP-IDH was not different. NADP-IDH (forward), reverse H^+-Thase, and CS were all significantly higher ($P < 0.05$) in SOL.

3.2. Rat muscle

Enzyme activities in the rat were measured in whole tissue homogenates of the three fiber types. All enzymes demonstrated a significant ($P < 0.05$) main effect for muscle type. In order to correct for the effects of differing mitochondrial contents among the fiber types, all enzyme activities were normalized to the mitochondrial marker citrate synthase activity (Table 2). After normalization, the SV (FG) muscle NAD-IDH specific activity was two-fold ($P < 0.05$) higher than SOL (SO) fibers. Forward H^+-Thase activity, normalized to CS, was much greater in rat red muscle fibers, having roughly 2.4- to 2.8-fold ($P < 0.05$) higher activities than white. Note that the DV (FOG) fibers fall between the two other fiber

Table 2
Enzyme activities of rat fiber types normalized to citrate synthase

Muscle (Type)	CS	NAD-IDH/CS	NADP-IDH/CS (forward)	Forward H^+-Thase/CS	Reverse H^+-Thase/CS
SOL (SO)	15.67 ± 0.94	4.65 ± 0.28	107.3 ± 4.95	0.42 ± 0.05	8.90 ± 0.70
DV (FOG)	16.34 ± 1.6	7.15 ± 0.50[a,b]	88.1 ± 8.6[b]	0.48 ± 0.08[b]	10.31 ± 1.05
SV (FG)	5.81 ± 0.78	9.74 ± 1.21[a]	39.6 ± 5.9[a]	0.17 ± 0.07[a]	12.40 ± 1.8

Values are means ± SE.
CS activity is μmol g^{-1} min^{-1}.
Other values represent (enzyme activity/CS activity) × 100.
[a] Significantly ($P < 0.05$) different than SOL.
[b] Significantly ($P < 0.05$) different than SV.

types, either significantly ($P < 0.05$) different than both, or similar to red muscle. Reverse H^+-Thase showed no significant differences between groups.

4. Discussion

The present study investigated quantitative differences in the enzymes of the proposed isocitrate dehydrogenase substrate cycle in different skeletal muscle fiber types.

In both GRAC and SOL, the activities of reverse NADP-IDH and forward H^+-Thase in skeletal muscle appear kinetically competent to support cycling, especially in Type I muscle mitochondria. These results support the work on heart mitochondria done previously [6]. This proposition is further strengthened when one considers the function of the cycle, i.e., greater control sensitivity. This cycle would function during high cellular energy states, when flux through the TCA cycle is low. Thus, cycling would be important when the NAD-IDH rate is kinetically inhibited by low ADP, and the forward H^+-Thase is energetically promoted by a high $\Delta\Psi$ across the mitochondrial inner membrane.

The higher specific activity of NAD-IDH in mitochondria from IIb muscle, along with lower forward H^+-Thase, in light of the higher activities of all other measured putative respiratory enzymes in Type I muscle mitochondria [13], suggests that Type IIb mitochondria may rely on NAD-IDH as a single locus of control of TCA cycle flux to a greater extent. This interpretation is consistent with the findings of Kushmerick et al. [14], who concluded that the control of respiration was more tightly linked to [ADP] in intact fast cat biceps than in the slow soleus.

The present study is the first we are aware of to measure many of these enzyme activities in both mitochondria from different skeletal muscle fiber types and in whole muscle homogenates. The mitochondrial specific activities for both NAD-IDH and CS agree closely with other studies performed in our lab [13], while the whole muscle activities are within the ranges reported by others [23,24]. NADP-IDH in the reverse direction is similar to those reported in mixed skeletal muscle [6], as were the values for the forward, non-energy linked H^+-Thase, reported in heart muscle [19]. Reverse H^+-Thase is slightly higher than those previously reported in heart muscle [6]. The measured forward transhydrogenase rate represents the non-energy linked rate. Sazanov and Jackson [2] report an energized rate of approximately 40 nmol mg^{-1} min^{-1} compared to a non-energy linked rate of 7 nmol mg^{-1} min^{-1} in heart submitochondrial particles, which is nearly identical to the rate in our study. Assuming that the energized transhydrogenase is amplified in skeletal muscle to the same extent as heart, the energized forward H^+-Thase for our tissues would be nearly identical to the reverse NADP-IDH rate in this study. In fact, given the very high energy state maintained by the mitochondria of Type II muscle [25], the amplification of forward H^+-Thase may be especially marked in fast muscle types.

Whole muscle NADP-IDH is within the range reported for skeletal muscle [26]. No data exists on the whole muscle transhydrogenase activities measured from tissue homogenates. However, dividing our whole tissue values by previously reported mitochondrial contents for different fiber types [13], brings our values into the ranges previously mentioned for mitochondrial specific activities.

The existence of the substrate cycle does not, however, exclude an attractive alternative use for NADPH generation through the forward NADP-IDH reaction: to buffer changes in the membrane potential through NADH production due to NADP-IDH and the reverse H^+-Thase reaction [10]. During times of extreme challenges to the cellular energy state, the flux through the TCA cycle may become greater than the catalytic potential of NAD-IDH, and mitochondrial Δp may fall enough to allow the H^+-Thase to shift in favor of NADH production, oxidizing NADPH and allowing NADP-IDH to generate NADPH. In this case, the high V_{max} of NADP-IDH (Table 1) could provide for the rapid production of NADPH to be transhydrogenated via the high activity of the reverse H^+-Thase and, in turn, allow for production of NADH at a high rate [6]. The oxidation of the NADH would be by NADH oxidase of the electron transport chain, extruding 10 protons [8]. Studies have shown that the transhydrogenation in the NADPH $\rightarrow$ NADH direction can account for only about 10% of respiration [4]. However, this 10% may be crucial in a time of extremely low cellular energy state.

A consequence of substrate cycle spinning is the dissipation of the mitochondrial Δp, as one proton is pumped per turn of the cycle. This influx of protons would contribute to the mitochondrial proton leak [27,28]. The energy to subsequently pump these protons out through the translocation sites of the electron transport chain would be needed and heat generated by the inefficiency inherent in these reactions. Therefore, higher rates of spin of the substrate cycle would produce more heat. Newsholme [9] asserts that the spin rate must balance the desired sensitivity and the possible hyperthermia caused by excessive spinning. However, the net result of one spin of this cycle is energy dissipation equivalent to the translocation of one proton (at the prevailing protonmotive force) across the inner mitochondrial membrane. Therefore, the proposed cycle should be able to maintain a high spin rate, with the resulting high sensitivity, without producing large amounts of heat.

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