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Hymenolepis diminuta: Mitochondrial transhydrogenase as an additional site for anaerobic phosphorylation

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Abstract

Employing adult *Hymenolepis diminuta* SMP and exogenous pyridine nucleotide-generating systems, reduced pyridine nucleotide-dependent net ^{32}P incorporation into ATP was examined. NADH supported rotenone-sensitive ^{32}P incorporation and this rate increased markedly with fumarate addition, in keeping with an active fumarate reductase. Interestingly, corresponding evaluations with NADPH did not result in detectable phosphorylation in the absence or presence of fumarate. However, with NAD addition, but without NAD generation, active NADPH-dependent phosphorylation occurred, thereby demonstrating mitochondrial transhydrogenase involvement, and ^{32}P incorporation increased significantly with fumarate addition. More importantly, in the presence of rotenone and both NADPH and NAD generation, significant net ^{32}P incorporation was noted, but was undetectable in the presence of DCCD or protonophores (e.g., niclosamide). Without NAD generation, minimal phosphorylation occurred. These data demonstrate that with ongoing NADPH and NAD generation, the *H. diminuta*, proton-translocating, mitochondrial transhydrogenase can serve as an additional anaerobic phosphorylation site. A model is presented.

Index Descriptors and Abbreviations: *Hymenolepis diminuta*; Cestode; Mitochondria; Submitochondrial particles; NADH oxidase; Fumarate reductase; NADPH → NAD transhydrogenase; Electron transport; Anaerobic phosphorylation site; ATP generation; NADPH → NAD reaction, NADPH + NAD → NADP + NADH; NADH → NADP reaction, NADH + NADP → NAD + NADPH; IM, mitochondrial inner membrane; SMP, submitochondrial particles; ATPase/synthase, adenosine triphosphatase/adenosine triphosphate synthase; EDTA, ethylenediaminetetraacetic acid; Tris–HCl, Tris (hydroxymethylaminomethane)–HCl; BSA, bovine serum albumin; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone

1. Introduction

In terms of its physiological energetics, the adult intestinal cestode, *Hymenolepis diminuta*, is predominantly anaerobic and, via the mitochondrial utilization of cytosolic malate, accumulates succinate as the major end product of glucose dissimilation (Fairbairn et al., 1961; Scheibel and Saz, 1966; Fioravanti and Saz, 1980). Malate, arising from CO_2 fixation into glycolytically formed phosphoenolpyruvate, serves as the substrate for a mitochondrial dismutation reaction that supports electron transport-coupled, site I-linked, net anaerobic phosphorylation (Scheibel and Saz, 1966; Scheibel et al., 1968; Saz et al., 1972; McKelvey and Fioravanti, 1984). NADP specific "malic" enzyme catalyzes the oxidative branch of this dismutation resulting in the production of pyruvate, CO_2, and reducing power in the form of NADPH (Prescott and Campbell, 1965; Saz et al., 1972; McKelvey and Fioravanti, 1984). However, the cestode electron transport system needs NADH as the reductant (Saz et al., 1972; Fioravanti, 1981). This need is satisfied by action of the mitochondrial, IM-associated pyridine nucleotide transhydrogenase (EC 1.6.1.1) that catalyzes hydride ion transfer from NADPH to NAD (NADPH → NAD reaction), thereby producing the needed NADH (Saz et al., 1972; Fioravanti and Saz, 1976; Fioravanti, 1981). Thereafter, the NADH-dependent, electron transport-coupled reduction of fumarate

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(arising from malate) to succinate completes the dismutation reaction with concomitant ATP generation (Scheibel and Saz, 1966; Scheibel et al., 1968; Saz et al., 1972).

The *H. diminuta* transhydrogenase catalyzes a reversible reaction with hydride ion transfer occurring on the matrix surface of the IM (Fioravanti and Saz, 1976; McKelvey and Fioravanti, 1985). Using isolated and everted IM vesicles, i.e., SMP, the *H. diminuta* transhydrogenase was found to catalyze both non-energy-linked and energy-linked NADH → NADP reactions; energy for the latter being derived from a proton gradient established by either electron transport-dependent NADH oxidation or ATP hydrolysis catalyzed by Mg^{2+}-dependent ATPase (Fioravanti et al., 1992; Park and Fioravanti, 2006). The ATP hydrolyzed/NADPH is about unity for the cestode ATP-dependent transhydrogenation (Park and Fioravanti, 2006). Moreover, using SMP, Mercer et al. (1999) demonstrated that with NADPH → NAD transhydrogenation, the *H. diminuta* enzyme catalyzes concomitant, transmembrane proton translocation from the mitochondrial matrix to the intermembrane space compartment.

The data supporting a proton gradient as the driving force for the *H. diminuta* energy-linked NADH → NADP reactions (Fioravanti et al., 1992; Park and Fioravanti, 2006), in conjunction with those demonstrating concomitant proton translocation with NADPH → NAD transhydrogenation, suggested that with NADH formation, the transhydrogenase serves as an additional site for anaerobic phosphorylation. In this regard, both bovine heart SMP and *Escherichia coli* membrane particles engage in transhydrogenase-dependent phosphorylation, in the virtual absence of electron transport activity, when catalyzing NADPH → NAD transhydrogenation (Van de Stadt et al., 1971; Donstov et al., 1972; Chetkauskaite and Grinius, 1979). The present studies were undertaken to evaluate the possibility of transhydrogenase-dependent phosphorylation in *H. diminuta*. Using *H. diminuta* phosphorylating SMP and substrate generating systems, the data presented demonstrate a transhydrogenase-dependent net phosphorylation in the virtual absence of electron transport, thereby indicating an additional site for anaerobic phosphorylation.

2. Materials and methods

Hymenolepis diminuta infections were maintained in either male or female Sprague–Dawley rats. Infection of animals and recovery of adult helminths were performed as described previously (Fioravanti, 1981). Cestode tissue was minced and subsequently homogenized in mitochondrial medium consisting of 250 mM sucrose, 15 mM EDTA (pH 7.5), and 10 mM Tris–HCl (pH 7.5). Cellular debris was removed by centrifugation at 482*g* for 10 min and mitochondria were isolated from the resulting supernatant fraction by centrifugation at 10,000*g* for 15 min. Isolated mitochondria were suspended in medium consisting of 250 mM sucrose and 10 mM Tris–HCl (pH 7.5). Thereafter, "heavy" mitochondria were recovered and washed by centrifugation at 17,000*g* for 10 min, suspended in 250 mM sucrose and stored at −20 °C for about 24 h (Fioravanti et al., 1992).

SMP isolation was accomplished after Hansen and Smith (1964) essentially as described by Fioravanti et al. (1992). The thawed suspension of "heavy" mitochondria was supplemented with the following to yield the final concentrations listed: 250 mM sucrose, 5 mM $MgSO_4$, 10 mM $MnCl_2$, 1 mM succinate, and 1 mM ATP. The suspension was maintained at pH 7.5 by the addition of a few drops of dilute NaOH. After 10 min, the suspension was sonically disrupted (6–15 s bursts with 1 min cooling intervals) using a Branson probe-type sonicator, equipped with a microtip, at a power setting of 22 W. The sonicated material was subjected to centrifugation at 17,000*g* for 6 min and SMP were obtained from the supernatant fraction by centrifugation at 100,000*g* for 40 min. Further recovery of SMP was achieved by repeating the sonication and centrifugation procedures on the 17,000*g* pellet obtained after the initial disruption of mitochondria. The SMP were suspended in 250 mM sucrose and 10 μM $MgSO_4$. Mitochondrial isolation and SMP preparation were performed at 4 °C.

Net phosphorylation was assayed by a modification of the procedure of Saz et al. (1972). For NADH-dependent phosphorylation, the 1 ml reaction volume contained SMP equivalent to 0.4–0.5 mg of protein, 0.5% BSA, 5 U of yeast hexokinase, 5 U of bovine liver glutamate dehydrogenase, and the following in μmol: glutamate, 3; Tris–HCl (pH 7.5), 50; $MgCl_2$, 10; ADP, 2; glucose, 3; sodium fluoride, 10; $KH_2{}^{32}PO_4$ (pH 7.5), 20 (2.3×10^4 dpm/μmol); NAD, 0.6, where indicated fumarate, 0.6; and rotenone 0.01. The reaction was started with the addition of NAD.

NADPH-dependent phosphorylation, in the presence or absence of fumarate, was measured using the system employed for NADH-dependent phosphorylation with the following exceptions: 0.24 μmol NADP replaced NAD, 5 U of yeast glucose-6-phosphate dehydrogenase and 3 μmol of glucose-6-phosphate were substituted for glutamate and glutamate dehydrogenase, respectively. The reaction was started with the addition of NADP.

Transhydrogenase-mediated, NADPH-dependent phosphorylation was measured employing the system for NADPH-dependent phosphorylation with the exceptions that, where indicated, the assay system contained 0.6 μmol NAD or 0.6 μmol of both NAD and fumarate. The reaction was started with the addition of NAD.

Transhydrogenase-dependent phosphorylation was measured employing the assay system for transhydrogenase-mediated NADPH-dependent phosphorylation with the exceptions that the system contained 0.01 μmol rotenone, and where indicated, 15 μmol pyruvate and 5 U of rabbit muscle lactate dehydrogenase. The reaction was started with the addition of NAD.

Rotenone was added to the assays in ethanolic solution such that ethanol was present at 1.5%, v/v. CCCP, FCCP, niclosamide, and DCCD were dispensed in the