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PURIFICATION AND PROPERTIES OF THE *ASCARIS* PYRUVATE DEHYDROGENASE COMPLEX

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Summary

The pyruvate dehydrogenase complex (pyruvate:lipoate oxidoreductase (decarboxylating and acceptor-acetylating), EC 1.2.4.1) has been isolated from *Ascaris* muscle mitochondria and purified to near homogeneity by differential centrifugation, $(NH_4)_2SO_4$ fractionation and calcium phosphate gel-cellulose chromatography. It is similar in shape, size and physical characteristics to pyruvate dehydrogenase complexes isolated from mammalian sources. It has an absolute dependence on CoA, NAD^+ and pyruvate for activity and is competitively inhibited by acetyl-CoA and NADH. However, much higher NADH/NAD^+ ratios are necessary to inhibit activity, suggesting regulation by the more reduced state of the pyridine nucleotide pool in *Ascaris* mitochondria.

Introduction

The parasitic nematode, *Ascaris lumbricoides* var. *suum* exhibits a predominantly anaerobic energy metabolism and accumulates a number of compounds as end products of carbohydrate fermentations [1,2]. These include succinate and a mixture of more reduced volatile fatty acids comprised of acetate, propionate, *n*-valerate, 2-methylbutyrate, tiglate and 2-methylvalerate. Energy is generated within *Ascaris* mitochondria by the anaerobic dismutation of malate [3]. 'Malic' enzyme (malate dehydrogenase, decarboxylating) converts malate to pyruvate and CO_2 and generates reducing power in the form of NADH [4]. This NADH then reduces fumarate to succinate. The reduction is coupled to an electron transport-associated ATP synthesis, mediated by the fumarate reductase system [5,6]. Possibly, another source of intramitochon-

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drial reducing power also may arise from the oxidative decarboxylation of pyruvate to acetate. This additional reducing power might be coupled to either or both branched-chain fatty acid synthesis and additional energy generation. No other reactions are known in *Ascaris* mitochondria which would supply sufficient reducing equivalents to account for the quantities of saturated volatile acid formed. Propionyl-CoA condensation and 2-methylacetoacetate reductase activities have been reported to be present in *Ascaris* muscle mitochondria [7].

An NAD^+-linked, CoA-dependent pyruvate decarboxylation has been identified in preparations of *Ascaris* mitochondria [8,9]. In aerobic tissues, this overall reaction is catalyzed by a multienzyme complex of about 9 million molecular weight and consists of three different enzymes: pyruvate dehydrogenase (pyruvate:lipoate oxidoreductase (decarboxylating and acceptor-acetylating, EC 1.2.4.1), dihydrolipoyl dehydrogenase (EC 1.6.4.3) and dihydrolipoyl transacetylase (EC 2.3.1.12). The present study was designed to isolate and characterize the pyruvate-oxidizing activity identified in *Ascaris* mitochondria and, more importantly, to compare its regulation to pyruvate dehydrogenase complexes isolated from aerobic tissues.

Materials and Methods

Ascaris lumbricoides var. *suum* muscle strips were obtained by dissection and mitochondria were isolated as previously described [3]. Mitochondrial pellets were resuspended to a concentration of approximately 15 mg protein/ml in 100 mM potassium phosphate buffer (pH 7.4) which contained 0.1 mM EDTA. The suspension was stored frozen at −20°C until further use. For enzyme purification, the frozen mitochondrial preparations were resuspended uniformly with the aid of a Teflon-glass homogenizer and sonicated four times for 30-s intervals with 15-s cooling periods employing a Bronwill sonifier fitted with a microtip at a power setting of 30 W.

Overall activity of the pyruvate dehydrogenase complex was measured routinely by monitoring NADH formation spectrophotometrically at 340 nm and 22°C according to the procedure of Linn et al. [10]. The standard assay system contained in a final volume of 1 ml: 50 mM potassium phosphate buffer (pH 7.4), 1 mM $MgCl_2$, 0.2 mM NAD^+, 3 mM cysteine-HCl, 1 mM thiamine pyrophosphate, 0.2 mM CoA, 3 mM pyruvate. The enzyme solution was diluted to produce an absorbance change of not more than 0.1 absorbance unit/min and the reaction was initiated by the addition of pyruvate after a 2 min preincubation period. Alternatively, the pyruvate dehydrogenase complex activity was assayed by measuring the rate of $^{14}CO_2$ evolution from [1-^{14}C]pyruvate according to Schwartz and Reed [11]. Both assays yielded similar results. Apparent K_m values for substrates were determined from Lineweaver-Burk plots [12] at saturating concentrations of cosubstrates. K_i values for NADH and acetyl-CoA inhibition were estimated from Dixon plots [13].

Lipoamide dehydrogenase activity was assayed in the direction of NAD^+ formation according to Sakurai et al. [14] except that NAD^+ was omitted from the assay system. Transhydrogenase and transacetylase activities were deter-

mined according to Köhler and Saz [15] and Reed and Willms [16], respectively. The pyruvate dehydrogenase component of the complex was measured by monitoring the reduction of ferricyanide at 420 nm as described by Schwartz and Reed [11].

For determinations of cofactor specificity and ATP inactivation purified enzyme preparations (10 mg/ml) were mixed with 100 mM potassium phosphate buffer, pH 7.4, (0.1 ml enzyme:4.9 ml buffer) and centrifuged at 200 000 × *g* for 1 h. The pellet was then suspended in 100 mM potassium phosphate buffer (pH 7.4) for cofactor analysis.

ATP inactivation of pyruvate dehydrogenase complex was studied in purified enzyme preparations by incubation at 22° C in 100 mM Tris-HCl, containing 10 mM dithiothreitol, 1 mM $MgCl_2$, 20 mM NaF and 0.01—1 mM ATP. At appropriate intervals, aliquots were removed and assayed immediately for pyruvate dehydrogenase complex activity.

Acetoin formation by the purified enzyme was measured directly. The incubation mixture contained 100 mM potassium phosphate buffer (pH 6.0), 0.04 mM thiamine pyrophosphate, 0.4 mM $MnCl_2$, 100 mM pyruvate and 0.2 mg enzyme in a final volume of 1 ml. After 60 min at 37° C the mixture was deproteinized according to Somogyi [17] and assayed for acetoin colorimetrically [18].

Sedimentation velocity studies were conducted in a Beckman Model E ultracentrifuge equipped with the RTIC unit. Sedimentation coefficients were corrected to standard conditions (water at 20° C) and extrapolated to infinite dilution. The partial specific volume was assumed to be 0.73 cm^3/g.

The purified complex was examined electron microscopically by suspending in 100 mM potassium phosphate buffer (pH 7.4) at a concentration of 1 mg protein/ml buffer. The suspension was placed on parlodion/carbon-coated grids, a drop of 1% ammonium molybdate (pH 7.2) was added, and excess stain was removed. After drying, the grids were viewed with an Hitachi HS-8 microscope.

Protein was determined according to Lowry et al. [19] with bovine serum albumin as the standard. Calcium phosphate gel suspended on Whatman standard grade cellulose powder was prepared as described by Price and Greenfield [20].

Pyridine nucleotides and coenzyme A were purchased from P and L Biochemicals (Milwaukee, WI). Enzymes were obtained from Boehringer Mannheim Corp. (New York, NY). The acetoin dimer (Aldrich, Milwaukee, WI) was washed with ether and dried in vacuo at room temperature. Acetaldehyde (Mallinckrodt, St. Louis, MO) was redistilled immediately prior to use. Calcium phosphate gel was a product of BioRad Laboratories, Richmond, CA, and enzyme grade ammonium sulfate was obtained from Schwarz/Mann, Orangeburg, NY.

Results

Purification of the Ascaris pyruvate dehydrogenase complex

Preparations of *Ascaris* mitochondria could be stored at −20° C for at least 30 days with little loss of activity. Pyruvate dehydrogenase complex activity

was not detected in intact mitochondria, but appeared in preparations which had been frozen and thawed. Subsequent sonication of this preparation resulted in up to a two-fold increase in activity. NADH oxidase interfered with the spectrophotometric assay in fresh preparations, but this activity was labile to freezing and storage and not significant in aged preparations. All operations in the purification were carried out at 0—4°C.

The sonicated mitochondrial suspension was centrifuged for 30 min at 18 000 × *g* and the pellet discarded. The supernatant was centrifuged at 150 000 × *g* for 90 min and the pellet was resuspended in 20 ml of 100 mM potassium phosphate buffer (pH 7.4) which contained 0.1 mM EDTA, 0.1 mM thiamine pyrophosphate, 1 mM $MgCl_2$, and 1 mM dithiothreitol (buffer A). The low and high-speed centrifugation series was then repeated.

Ammonium sulfate fractionation. This preparation was fractionated further by the addition of saturated ammonium sulfate (pH 7.4) which contained 10 mM β-mercaptoethanol. The bright yellow precipitate obtained between 0.45 and 0.53 saturation was collected by centrifugation at 10 000 × *g* for 20 min and was dissolved in 4 ml of buffer A. Any insoluble material was removed by centrifugation at 18 000 × *g* for 30 min.

Calcium phosphate gel-cellulose chromatography. The enzyme complex was applied to a calcium phosphate gel-cellulose column (1.0 × 2.0 cm) which previously had been equilibrated with buffer A. The column was washed with 1% ammonium sulfate in buffer A until the absorbance at 280 nm approached zero. The enzyme complex remained as a bright yellow band at the top of the column and was eluted, as a single sharp peak, with a solution of 5% ammonium sulfate in buffer A. Fractions containing the highest activity were pooled, centrifuged at 150 000 × *g* for 2 h, and the pellet was resuspended in buffer A.

Concentrated solutions of the purified enzyme complex (10 mg/ml) were yellow, opalescent, highly fluorescent and lost less than 20% of their activity after storage for 30 days at −20°C. More dilute solutions of the enzyme could be stabilized by the addition of 1% bovine serum albumin. If necessary, enzyme solutions were centrifuged at 18 000 × *g* for 30 min to remove insoluble material before further use. Routinely the purification was completed in one day and final specific activities ranged between 1.2 and 2.0 μmol/min per mg protein (Table I).

TABLE I

PURIFICATION OF THE PYRUVATE DEHYDROGENASE COMPLEX FROM *ASCARIS* MUSCLE MITOCHONDRIA

Step		Volume (ml)	Activity ($\mu mol \cdot min^{-1}$)	Protein (mg)	Specific activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	Recovery (%)
I	Mitochondrial sonicate	120	36.6	960	0.038	100
II	Centrifugation	20	35.6	96	0.38	97
III	Ammonium sulfate	5	30.3	27	1.11	83
IV	Calcium phosphate gel-cellulose	1	20.5	14	1.42	56

Homogeneity and size of the Ascaris pyruvate dehydrogenase complex

Studies of the purified complex yielded sedimentation coefficients of 76 ± 2 S ($n = 3$) and suggested that the preparations were 90—95% homogenous. The sedimentation coefficient was independent of protein concentration from 0.5 mg/ml to 6 mg/ml (Fig. 1). Below 0.5 mg/ml a slight decrease in the $s_{20,w}$ was recorded and slower migrating components were observed, implying that the complex underwent partial dissociation.

Purified preparations of the complex were negatively stained with ammonium molybdate and examined under the electron microscope (Fig. 2). Images with a diameter of about 300 Å diameter were observed which were similar in appearance and size to those of the mammalian pyruvate dehydrogenase complex. Many of the large particles had smaller particles attached to their periphery, again suggesting an organization similar to other pyruvate dehydrogenase complexes.

Enzymic activities associated with the Ascaris pyruvate dehydrogenase complex

Maximal NAD^+ reduction was dependent on the presence of CoA, thiamine pyrophosphate, pyruvate and cysteine. However, the omission of thiamine pyrophosphate from the assay mixture still resulted in a substantial amount of activity (36%), implying that significant thiamine pyrophosphate remained

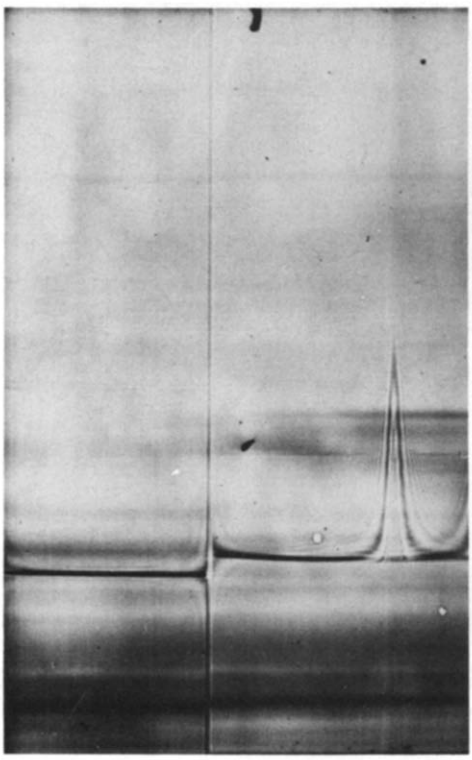

Fig. 1. Sedimentation pattern obtained with the purified pyruvate dehydrogenase complex after 16 min at 24 000 rev./min, at 20°C using Schlieren optics. The protein concentration was 2.5 mg/ml in 100 mM potassium phosphate (pH 7.4) buffer, 0.1 mM EDTA.

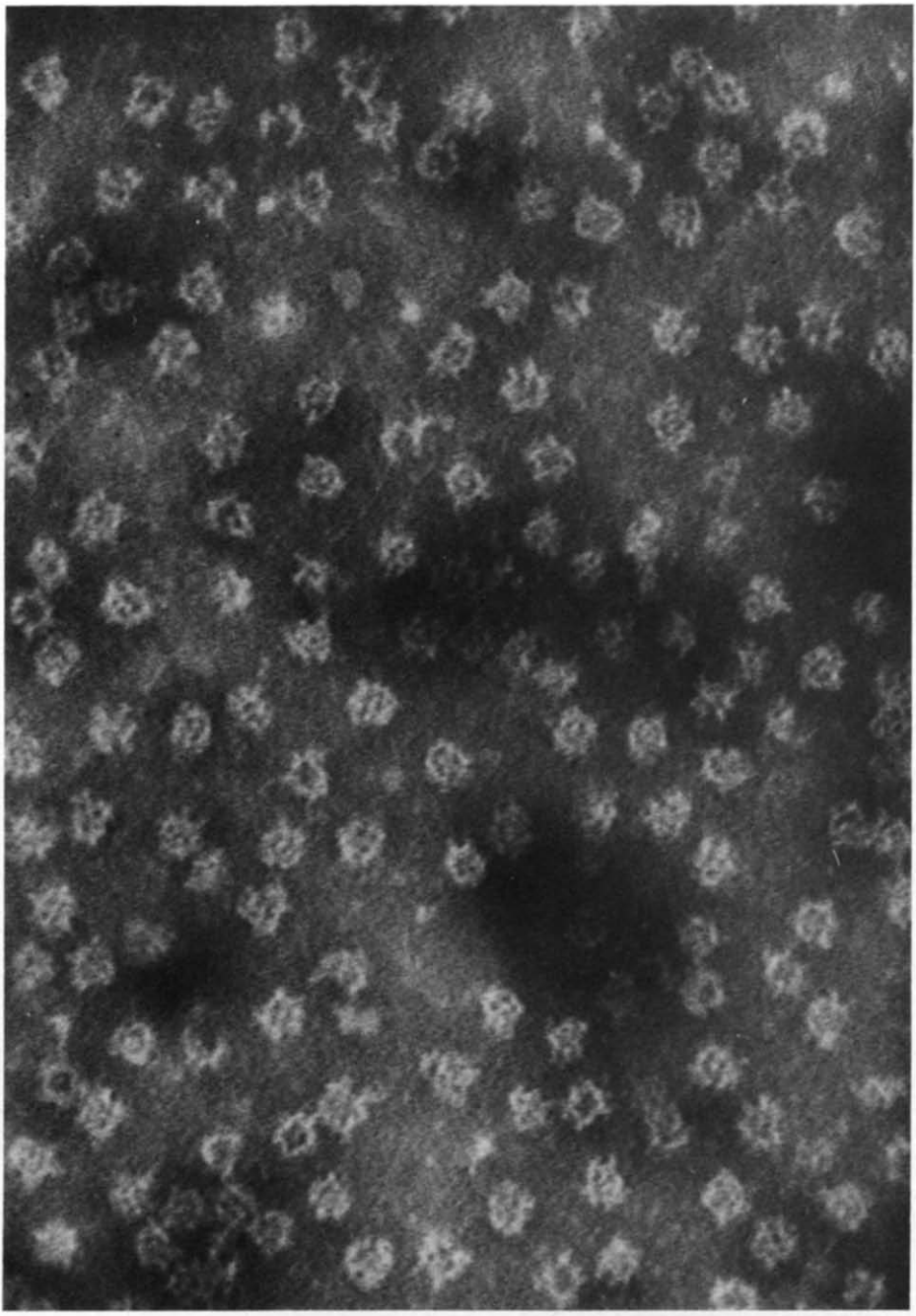

Fig. 2. Electron micrograph (X140 000) of the *Ascaris* pyruvate dehydrogenase complex negatively stained with ammonium molybdate.

bound to the complex during the preparation of the enzyme for studies of cofactor dependence. Unfortunately, further attempts to remove this cofactor by dialysis against a variety of buffers resulted in a substantial, irreversible,

TABLE II

ENZYMIC ACTIVITIES ASSOCIATED WITH THE *ASCARIS* PYRUVATE DEHYDROGENASE COMPLEX

Assay	Specific activity (μmol · min^{-1} · mg^{-1} protein)	
	Crude *	Purified
Pyruvate: NAD^+ reductase (overall)		
a. $^{14}CO_2$ evolution	0.04	1.67
b. NAD^+ reduction	0.03	1.53
Pyruvate: $K_3Fe(CN)_6$ reductase		0
Transacetylase		4.3
Lipoamide dehydrogenase	0.80	22.0
NADH: NAD^+ transhydrogenase	0.42	7.1

* Sonicated mitochondria centrifuged at 18 000 × *g* for 30 min and the supernatant used as the source of enzymes.

inactivation of enzymic activity. $NADP^+$ could not replace NAD^+ and α-ketoglutarate could not replace pyruvate in the reaction. In addition to this overall activity, the complex also catalyzed a lipoate-CoA transacetylation, as well as the oxidation of NADH by lipoamide (Table II). Attempts to demonstrate a distinct pyruvate decarboxylase activity using either ferricyanide or dichlorophenol indophenol as electron acceptor have yielded negative results, which contrasts with other pyruvate dehydrogenase complexes studied to date [11,21,22]. The complex also contained substantial NADH:NAD^+ transhydrogenase activity. Fractionation experiments have indicated that this activity is associated with the dihydrolipoyl dehydrogenase component of the complex [23]. During the initial steps in the purification, substantial amounts of dihydrolipoyl dehydrogenase may be dissociated from the complex and, in fact, the initial 150 000 × *g* supernatant has been used as a source for the purification of this enzyme [23]. However, the addition of purified *Ascaris* dihydrolipoyl dehydrogenase [23] to the assay mixture did not stimulate the overall pyruvate dehydrogenase complex activity, suggesting that dihydrolipoyl dehydrogenase is present in excess.

The purified complex also catalyzed the formation of acetoin from either acetaldehyde (0.07 μmol acetoin formed · h^{-1} · mg^{-1} protein) or pyruvate (0.22 μmol acetoin formed · h^{-1} · mg^{-1} protein) but the rate was much greater with both acetaldehyde and pyruvate (3.73 μmol acetoin formed · h^{-1} · mg^{-1} protein). The reaction with both substrates was linear for up to 3 h and had a pH optimum of about 6.0.

Kinetic characteristics of the purified Ascaris pyruvate dehydrogenase complex

Michaelis and inhibitor constants for the substrates and inhibitors of the *Ascaris* pyruvate dehydrogenase complex were determined. The apparent K_m values for pyruvate, NAD^+ and CoA were 0.185 mM, 0.01 mM and 0.005 mM, respectively. Inhibition by acetyl-CoA was competitive with respect to CoA, with a K_i of 62 μM (Fig. 3A). In addition, 1 mM propionyl-CoA slightly inhibited (20%) enzyme activity, while 1 mM butyryl-CoA and 1 mM tiglyl-CoA (*cis*-2-methylcrotonyl-CoA) had no effect. Inhibition by NADH was

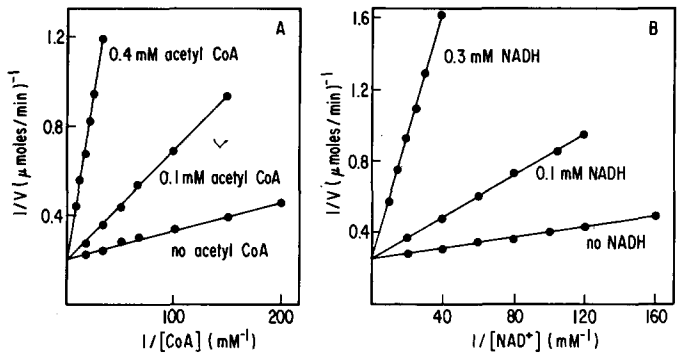

Fig. 3. (A) Double-reciprocal plot of velocity as a function of CoA concentration in the presence and absence of acetyl-CoA. (B) Double-reciprocal plot of velocity as a function of NAD^+ concentration in the presence and absence of NADH. The reaction mixtures contained in 1 ml: 50 mM potassium phosphate buffer (pH 7.4), 1 mM $MgCl_2$, 0.1 mM NAD^+, 3 mM cysteine-HCl, 1 mM thiamine pyrophosphate. 0.2 mM CoA, 3 mM pyruvate and enzyme.

competitive with respect to NAD^+, with a K_i of 25 μM (Fig. 3B). More importantly, enzymic activity was markedly controlled by the NAD^+/NADH ratio (Fig. 4). The physiological significance of this phenomenon is discussed below.

ATP inhibition of the Ascaris pyruvate dehydrogenase complex

Repeated attempts to demonstrate a phospho-dephospho form of regulation similar to that operating in mammalian tissues were unsuccessful. Incubation of the purified complex with 0.01—1 mM ATP and 1 mM $MgCl_2$ in the presence and absence of NaF had no effect on pyruvate dehydrogenase complex activity when assayed either spectrophotometrically or by monitoring $^{14}CO_2$ evolution from [1-^{14}C]pyruvate. In addition, during incubations of the

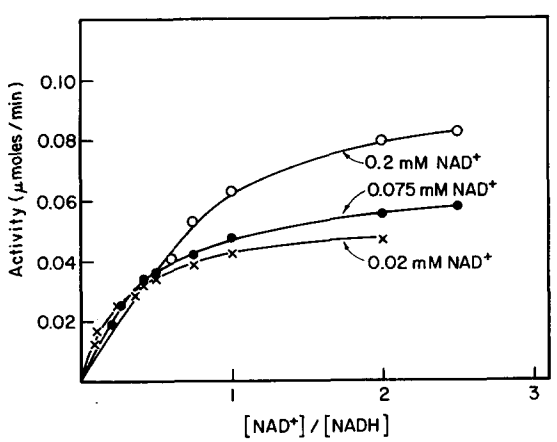

Fig. 4. The effect of changes in the NAD^+/NADH ratio on the activity of the *Ascaris* pyruvate dehydrogenase complex. In addition to NAD^+ and NADH, the reaction mixture contained in 1 ml: 50 mM potassium phosphate buffer (pH 7.4), 1 mM $MgCl_2$, 3 mM cysteine, 1 mM thiamine pyrophosphate, 0.2 mM CoA, 3 mM pyruvate and enzyme.

purified complex (8 mg) with 0.1 mM [γ-^{32}P]ATP and 1 mM $MgCl_2$ for 1 h no radioactivity was incorporated into a trichloroacetic acid-precipitable fraction. Studies are currently underway using intact and sonicated preparations of isolated mitochondria to ascertain whether these observations are physiological or whether they result from some alteration of the complex during isolation, i.e. loss of the pyruvate dehydrogenase kinase.

Discussion

Ascaris mitochondria contain substantial quantities of pyruvate dehydrogenase complex, amounting to about 2.5% of the total mitochondrial protein. The *Ascaris* complex is similar in many respects to pyruvate dehydrogenase complexes isolated from a number of other sources [10,21,26]. For example, the sedimentation coefficients reported in the present study are similar to those reported for the yeast [27] and mammalian complexes [21], while the catalytic and kinetic parameters also are similar to their mammalian counterparts [21,26,27]. In contrast to other systems [11,21], attempts to assay pyruvate dehydrogenation directly by coupling to artificial electron acceptors, such as ferricyanide, have been unsuccessful. Perhaps electron flow is more tightly coupled in the helminth system. This possibility is of particular interest in view of the postulated physiological role of the pyruvate dehydrogenase complex in the formation of 2-methylbutyrate, a major product of *Ascaris* fermentation. Evidence has been reported that 2-methylbutyrate is synthesized in *Ascaris* muscle by condensation of acetyl-CoA with propionyl-CoA [28,29]. The resulting 2-methylacetoacetyl-CoA then would undergo a reduction followed by a dehydration to tiglyl (*cis*-2-methylcrotonyl)-CoA, another product of *Ascaris* fermentation. A final reduction of the double bond would form 2-methylbutyryl-CoA. 2-Methylacetoacetate reductase has been partially characterized and shown to be NADH linked [7]. On the other hand, the reduction of tiglyl-CoA could not be demonstrated employing numerous natural and artificial electron donors. Obviously, the reactions of the pyruvate dehydrogenase complex could supply NADH for the 2-methylacetoacetate reduction, but whether or not it can couple directly with the reduction of tiglyl-CoA remains to be determined.

The pyruvate dehydrogenase moiety of the *Ascaris* pyruvate dehydrogenase complex does catalyze acetoin formation from pyruvate or acetaldehyde. In fact, the intact nematode or muscle homogenates form substantial amounts of acetoin [30]. The present study suggests that the pyruvate dehydrogenase complex may be responsible for this synthesis, a property shared with pyruvate dehydrogenase complexes from all other sources examined [27,31—33].

The activity of the *Ascaris* pyruvate dehydrogenase complex is markedly affected by CoA/acetyl-CoA and NAD^+/NADH ratios. The free NAD^+/NADH ratio in mammalian liver mitochondria is approximately 10 [34]. In contrast with the corresponding mammalian system, at this ratio the *Ascaris* pyruvate dehydrogenase complex would be maximally active and unaffected by the redox state of the nicotinamide nucleotide pool (see Fig. 4). However, *Ascaris* energy metabolism is anaerobic and the free intramitochondrial nucleotide pool is maintained in a much more reduced state. Barrett [35] and Barrett and Beis

[36] have calculated the free $NAD^+/NADH$ ratio in *Ascaris* mitochondria to be in the range of 0.07—0.7 by using the equilibrium constant of the 'malic' enzyme and estimating the mitochondrial concentrations of malate and pyruvate. Within this range the *Ascaris* pyruvate dehydrogenase complex is maximally regulated; small changes in nucleotide concentration ratio would lead to large changes in pyruvate dehydrogenase complex activity. If this is the case then the pyruvate dehydrogenase complex could play a major regulatory role in the synthesis of volatile fatty acids.

Whether or not ATP can be generated as a consequence of the further utilization of the acetyl-CoA arising from the pyruvate dehydrogenase complex activity remains to be determined. The anaerobically functioning *Ascaris* mitochondrion possesses an incomplete tricarboxylic acid cycle, and aconitase and isocitrate dehydrogenase are only barely detectable [37]. Although acetate is a product of *Ascaris* metabolism, its formation may not be associated with nucleotide triphosphate formation as attempts to demonstrate acetate thiokinase activity in *Ascaris* have been unsuccessful (Komuniecki, R. and Saz, H., unpublished data). However, conservation of the thioester bond energy may occur through the action of an acyl-CoA transferase. That such a transferase is present in *Ascaris* muscle mitochondria and functions in the generation of ATP during the formation of propionate is indicated by the preliminary report of Saz et al. [38]. Alternatively, the reducing power generated by pyruvate oxidation could potentially couple to the fumarate reductase system and result in additional ATP synthesis.

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