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Pyruvate Dehydrogenase Complex From Ribbed Mussel Gill Mitochondria

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ABSTRACT The pyruvate dehydrogenase complex has been demonstrated in high speed pellet preparations from sonicated ribbed mussel gill mitochondria. The activity of the complex is inhibited by low chloride (< 100 mM) concentrations, EDTA (1 mM), succinate, ATP, and NAD/NADH ratios below 4. Inhibition by EDTA is relieved by addition of 10 mM $MgCl_2$–1 mM $CaCl_2$. ATP inhibition was enhanced by NaF and reversed by high Mg^{++} concentrations in the absence of NaF. Pyruvate and thiamine pyrophosphate inhibited the inactivation by ATP. The nonhydrolyzable ATP analog AMP–PNP caused inhibition of the overall catalytic activity that was identical to ATP. Factors involved in the ATP inhibition and Mg^{++} reversal are lost with freezing or cold storage. Preliminary results using γ-^{32}P-ATP indicate that a protein kinase that phosphorylates the α subunit of E_1 (pyruvate dehydrogenase) from the mammalian PDC is associated with the gill PDC. The activity of the complex may be regulated by a phosphorylation/dephosphorylation mechanism and by the relative levels of substrates, products, and other metabolites in the mitochondria.

Most estuarine bivalve mollusc tissues accumulate high levels of intracellular amino acids in response to hyperosmotic (see Pierce, '82) or anaerobic stress (deZwaan, '83). Accumulation of alanine in mussel tissues is transaminase dependent (Bishop et al., '81; Greenwalt and Bishop, '80; deZwaan et al., '83a,b). In ribbed mussel gills, this increase in alanine levels requires action of alanine aminotransferase activity, which is localized in the mitochondria (Paynter et al., '84). To control the accumulation of the high levels of alanine (0.1–0.2 M) during salt stress, turnover of pyruvate within mitochondria of these mussel tissues must be acutely regulated. Preliminary results (manuscript in preparation) suggest an acute inhibition of pyruvate dehydrogenase complex (PDC) to shunt mitochondrial pyruvate toward alanine. This inhibition of PDC is apparently coordinated with inhibition of the mitochondrial glycine cleavage enzyme to cause coordinate glycine accumulation (Ellis et al., '85). From other studies, it appears that acute regulation or modification of PDC activity may also occur in bivalves (Ho and Zubkoff, '82, '83; Kluytmans et al., '78) and helminth parasites (Komuniecki et al., '81; Rew and Saz, '74; Saz, '81) to account for anaerobic production of acetate from carbohydrate-derived pyruvate.

Pyruvate dehydrogenase complex is a multienzyme complex within the mitochondrion that catalyzes reaction of pyruvate with CoA and NAD to form acetyl CoA, CO_2, and NADH (see Reed, '74). Weiland ('83) has recently reviewed the structure and regulation of mammalian PDC. It is regulated primarily by an ATP-dependent phosphorylation/dephosphorylation (protein kinase–phosphatase) mechanism that is mediated by Ca^{++}, Mg^{++}, and/or other intracellular effectors. Komuniecki et al. ('79, '83) have investigated PDC from muscle tissue mitochondria of the "anaerobic" parasitic roundworm, *Ascaris*. Properties of this purified *Ascaris* enzyme appear to resemble those of the mammalian complex in general kinetic terms including the protein kinase–phosphatase regulatory characteristics.

Although a small amount of PDC has been reported in mitochondria from sea mussel

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tissues (Addink and Veenhof, '75), there are no studies on the regulatory or other properties of this important enzyme in molluscs. In fact, pyruvate added to coupled mollusc gill mitochondria failed to stimulate oxygen consumption (Burcham et al., '84). Questions have arisen as to the amount of enzyme in molluscan tissues and whether or not regulation of PDC was similar to complexes from other animals. In this report, we describe a convenient method for preparation and assay of PDC from bivalve tissue mitochondria, some kinetic properties of the complex and some preliminary evidence for regulation of this activity.

MATERIALS AND METHODS

Ribbed mussels (*Modiolus demissus*) were obtained from Northeast Marine Environmental Institute (Monument Beach, MA). The animals were maintained as described by Greenwalt and Bishop ('80). Except where noted, all reagents were purchased from Sigma (St. Louis, MO). Enzyme-grade ammonium sulfate and sucrose were obtained from Schwarz–Mann (Orangeburg, NY). 1-^{14}C-Pyruvate was obtained from New England Nuclear Corp.

The enzyme complexes were partially purified from a mitochondrial lysate by a series of differential centrifugations (Komuniecki et al., '79). Mitochondria were prepared as described by Paynter et al. ('84) and sonicated using a Branson sonifier on a setting of "5" with the intermediate size probe. Small batches (1–2 ml) of mitochondrial suspension were sonicated three times for 10 seconds on ice with 10 seconds between each sonic treatment. The sonicated suspension was centrifuged for 30 minutes at 20,000 g to remove unbroken mitochondria and debris. The supernatant fluid was centrifuged at 150,000 g for 90 minutes. The pellet from this centrifugation was resuspended in mitochondrial isolation buffer, then recentrifuged at 20,000 g for 30 minutes. The supernatant fluid was then centrifuged a second time at 150,000 g for 90 minutes. The resulting pellet was resuspended in mitochondrial isolation buffer and recentrifuged for 30 minutes at 20,000 g. The supernatant fluid from this final centrifugation constituted the enzyme preparation used for the preliminary studies described in this paper.

The enzyme activity was assayed both radiometrically and spectrophotometrically during preparation (Komuniecki et al., '79). The radiometric assay consisted of the incubation of 1-^{14}C-pyruvate (0.5 μCi) in a reaction mixture containing 2.5 mM pyruvate, 0.1 mM NAD, 0.1 mM thiamine pyrophosphate (TPP), 0.1 mM CoA, 1.0 mM dithiothreitol (DTT), and 100 mM Tris-HCl in 1 ml. The reaction was stopped after 1 hour with 2N HCl and the evolved CO_2 collected in a filter paper trap with 100 μl of 1 M hyamine hydroxide in methanol. This assay was used in the early phase of the purification where the spectrophotometric assay was impossible. The spectrophotometric assay employed the same reaction mixture (in 1 ml) and the production of NADH (A_{340}) was measured in a recording spectrophotometer. α-Ketoglutarate dehydrogenase (KGDC) was assayed using the same reaction mixture by substituting α-ketoglutarate for pyruvate. Apparent Michaelis constants for substrates were generated using Cleland's ('79) kinetic analysis.

ATPases were assayed by incubation with 5mM phosphoenolpyruvate (PEP), 5 units pyruvate kinase, 5 units lactate dehydrogenase, 70 μM NADH, and 1 mM ATP in 100 mM Tris HCl (pH 8.3). NADH oxidation was measured in a spectrophotometer (A_{340}). NADH oxidase activity was measured by incubation of enzyme with 70 μM NADH in 100 mM Tris HCl, pH 8.3.

The molluscan PDC was incubated with γ-^{32}P-ATP in an attempt to demonstrate covalent modification of a component of the complex directly. A sample (4 mg) was incubated with 100 mM Tris HCl (pH 8.3), 1 mM DTT, 10 μM TPP, and 200 μM ATP (specific activity = 300 μCi/μmole ATP) in 1 ml. Specific PDC protein kinase inhibitors pyruvate (5 mM) and TPP (200 μM) were also included in experimental incubations. We incubated 20 μg of purified mammalian PDH (E_1) with 4 mg of the molluscan prep in the same incubation mixture without the kinase inhibitors. Incubations were stopped by addition of 0.5 ml 2× sample buffer (Laemmli, '70) and heated immediately in a boiling water bath for 1 minute. Samples (approximately 0.5 mg protein and 7.5 μCi of radioactivity) were electrophoresed on 10% acrylamide gels according to Laemmli ('70). Low molecular weight standards (10,000–100,000) were obtained from Bio-Rad. The gels were stained with Coomassie brilliant blue in 25% isopropanol/10% acetic acid overnight, destained in 10% isopropanol/10% acetic acid, and fixed in 10% acetic acid/0.1% glycerol. The gels were dried on a Hoefer Model SE 540 slab gel

dryer and exposed at −70°C for 10 days in the dark using Cronex Lightning Plus intensifying screens and Kodak X-Omat AR (XAR-5) X-ray film.

Protein was estimated according to Lowry as modified by Miller ('59) or by the biuret method for mitochondrial suspensions (King, '67).

RESULTS AND DISCUSSION

In crude homogenates, mitochondrial suspensions, and mitochondrial lysates pyruvate dehydrogenase activity was demonstrated using the production of $^{14}CO_2$ from 1-^{14}C-pyruvate as the assay procedure. After ultracentrifugation, the complexes pelleting at 150,000 g were resuspended in buffer yielding a reasonably transparent sample for use with the spectrophotometric assay. This preparation showed CoA and NAD dependency using the radiometric procedure. Pyruvate and CoA dependency was demonstrated using the spectrophotometric method. The rate of the reaction increased with increasing enzyme concentration (Fig. 1). There was considerably more KGDC than PDC in gill mitochondria.

The activities of the PDC and KGDC with and without substrate or effectors are presented in Table 1. EDTA (1 mM) completely inhibited the PDC activity but did not affect the KGDC activity. Addition of 10 mM $MgCl_2$–1 mM $CaCl_2$ in buffer (Tris) restored full activity to preparations containing 1 mM EDTA. Arsenite (Ars) effectively inhibited both enzymes at a 1 mM. ATP addition caused almost complete inhibition of the PDC but only 35% inhibition of the KGDC. NaF had no effect on either enzyme in the absence of ATP. Addition of both ATP and NaF caused complete inhibition of PDC and a 40% reduction of the KGDC activity.

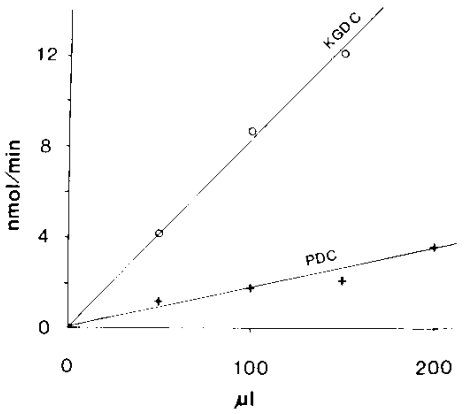

Fig. 1. Effect of amount of enzyme on the reaction rate of the pyruvate dehydrogenase complex (PDC) and the α-ketoglutarate dehydrogenase complex (KGDC) from the mitochondria of ribbed mussel gill tissue. Assay procedure is described in Materials and Methods.

Preliminary kinetic investigations on this crude PDC preparation indicated that the substrate K_m for the molluscan PDC were similar to those of the mammalian and *Ascaris* complexes. Standard error for all of the determinations was less than 20%. The apparent K_m values for pyruvate, CoA, and NAD were 300 µM, 5.8 µM, and 96 µM respectively. The apparent K_m for pyruvate seemed higher than those reported for many mammalian complexes whereas the values for CoA and NAD were similar (Blass and Lewis, '73; Roche and Cate, '77; Wieland, '83). The high apparent K_m for pyruvate with the gill PDC was comparable to the high

TABLE 1. Pyruvate dehydrogenase complex (PDC) and α-ketoglutarate dehydrogenase complex (KGDC) activities from mitochondria of ribbed mussel gill tissue

Reaction mixture	Activity (nmol/min/mg protein) PDC	KGDC
Complete	12.	62.
Without CoA	<0.01	<0.01
Without ketoacid	<0.01	<0.01
Plus Ars (1 mM)	0.01	0.60
Plus ATP (1 mM)	3.	40.
Plus NaF (10 mM)	12.	62.
Plus NaF (10 mM) + ATP (1 mM)	0.67	38.

*The assay procedure and reaction mixture containing 0.3 mg of protein are described in Materials and Methods. Where appropriate, enzyme activity was determined after a two minute preincubation with inhibitor. In the NaF + ATP assays, enzyme was incubated first with NaF for two minutes, then assayed. Dithiothreitol was present during all incubations except in the arsenite (Ars) experiment.

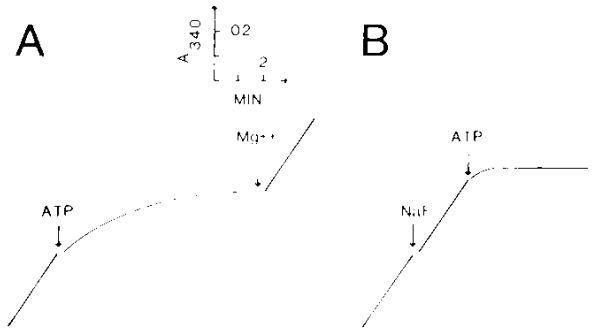

Fig. 2. Effect of ATP on the pyruvate dehydrogenase complex activity. (A) ATP and $MgCl_2$ were 1 mM and 10 mM, respectively. (B) NaF and ATP were 10 mM and 1 mM, respectively. ATP, $MgCl_2$, or NaF were added to the reaction mixture at the indicated intervals while the reaction rate was being monitored in the recording spectrophotometer.

apparent K_m for pyruvate (185 μM) reported for the *Ascaris* PDC (Komuniecki et al., '79).

In a series of experiments using the standard spectrophotometric assay procedure, alanine, aspartate, glycine, taurine, acetate, and proline at 100 mM concentrations had no inhibitory or activating effect on the catalytic activity of the molluscan PDC. Succinate (28 mM) inhibited the catalytic activity 50%. These properties were similar to those of the mammalian complex (Blass and Lewis, '73; Wieland, '83). Chloride had an inhibitory effect on the catalytic activity when low (< 100 mM) concentrations of KCl, NaCl, or choline chloride were added to the assay mixture. The I_{50} values were 63 mM (KCl), 63 mM (NaCl), and 40 mM (choline chloride). The catalytic activity was lost rapidly if the preparations were stored in chloride-containing buffers. NaF (50 mM) or sodium or potassium acetate (100 mM) showed no inhibitory effects. This molluscan complex also showed no changes in activity between pH 6.8 and 9.0 when assayed under standard substrate conditions.

ATP inhibition of the PDC was investigated more closely. ATP completely inhibited the PDC activity in a time-dependent fashion (Fig. 2A); this inhibition was reversed by subsequent addition of 10 mM $MgCl_2$ (Fig. 2A). When NaF was included in the reaction's mixture with ATP, the inhibition was more rapid and complete (Fig. 2B). This experiment supported the possibility of a kinase–phosphatase regulatory system for PDC.

To test the possibility that a Mg^{++}-stimulated ATPase was hydrolyzing ATP and thereby releasing the inhibitory effect of the ATP with the addition of Mg^{++}, the preparation was assayed for ATPase activity. The ATPase activity was relatively insignificant in the absence of additional Mg^{++} (0.50 nmol/min/mg protein), but increased to a high level (11.05 nmol/min/mg protein) in the presence of 10 mm Mg^{++}. This ATPase activity was insensitive to oligomycin (1–10 μg/ml) indicating that if the activity was the ATP synthase, the regulatory (oligomycin binding) subunit was lost during preparation. Given the level of ATP (1 mM) in the inhibition experiment (Fig. 2), this Mg^{++}-stimulated ATPase would have reduced the ATP level less than 5% and would not have affected ATP inhibition or the reversal of ATP inhibition with Mg^{++} addition.

To investigate the possibility of a kinase–phosphatase system further, other nucleotide phosphates were tested for inhibition of the PDC activity. Although GTP and ADP showed some effects at high (1 mM) concentrations, they showed no inhibitory effect in the range that ATP was effective (0.1–1 mM). The nonhydrolyzable ATP analogs, β,γ-methyleneadenosine 5′-triphosphate (AMP–PCP), and 5′-adenylylimidodiphosphate (AMP–PNP), were used in place of ATP. AMP–PCP inhibited only at very high (>1 mM) concentrations whereas inhibition by AMP–PNP was nearly identical to that exhibited by ATP at the same concentrations.

The effects of ATP and AMP–PNP were investigated further. High levels of both pyruvate and TPP have been shown to interfere with the protein kinase activity in PDC from mammalian mitochondria (Wieland, '83). Pyruvate and TPP concentrations were shown

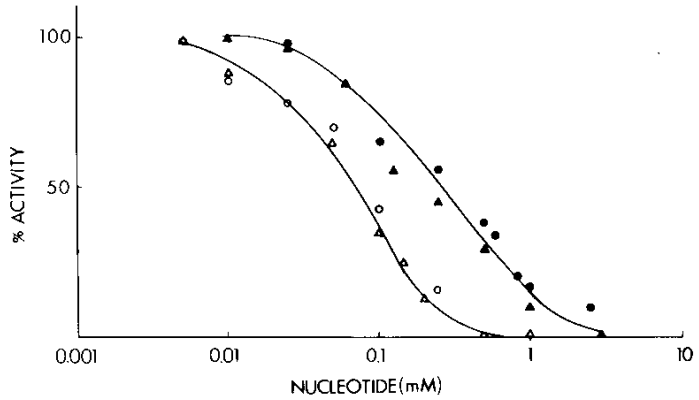

Fig. 3. The effect of TPP concentration on the inhibition of PDC activity by ATP (○,●) or AMP-PNP (△,▲). Activity was assayed as described in Materials and Methods in the presence of varying amounts of nucleotide at two different concentrations of TPP; 10 μM (○,△) and 100 μM (●,▲). TPP clearly blocks the inhibitory effects of ATP on the PDC activity.

to affect the inhibition of the gill PDC activity by ATP or AMP–PNP (Fig. 3). A ten-fold decrease in TPP concentration caused an approximate decrease in the ATP I_{50} value from 250 to 70 μM (see Fig. 3). Pyruvate also blocked the inhibitory effect of ATP but was not as effective as TPP. These results supported the hypothesis that the protein kinase was associated with the molluscan PDC and was inhibited by pyruvate and TPP. The inhibitory effect of AMP–PNP was curious and suggested that there was a direct effect of ATP and AMP–PNP on the PDC that might not be protein kinase–phosphatase related.

In order to test for the protein kinase activity in a more direct fashion, preparations were incubated with γ-^{32}P-ATP in the presence of the specific PDC protein kinase inhibitors, pyruvate, and TPP at concentrations that blocked ATP inhibition in the spectrophotometric assay; then the preparations were subjected to gel electrophoretic analysis (see Materials and Methods). Several protein bands were apparent in the gels of gill PDC preparations stained with Coomassie blue. Purified mammalian E_1 electrophoresed in a lane alone showed only two bands of 41 and 37 Kd. These protein bands were also apparent in the incubations containing the molluscan preparation and this preparation with mammalian E_1 added. Autoradiography of these gels revealed that several proteins were labelled by γ-^{32}P-ATP. A band in the gill PDC preparations at about 41 Kd which corresponded to the α subunit of the mammalian E_1 was labelled. In incubations containing mammalian E_1 mixed with the gill PDC preparation, the 41 Kd band was the major band labelled and contained significantly more radioactivity than samples without the E_1 added. This 41 Kd band was the α subunit of the E_1 component of mammalian PDC and the subunit that is phosphorylated in the mammalian (Barrera et al., 1972; Reed, 1974) and *Ascaris* (Komuniecki et al., 1983) complexes. No label was detected in a 37 Kd protein of either the purified E_1 or the gill PDC preparation. These experiments indicated that the preparation contained a protein kinase that would covalently modify the 41 Kd subunit of the mammalian PDH component of the complex. The faintly ^{32}P-labelled 41 Kd component in the gill PDC preparation may be the E_1 from the PDC complex preparation. Experiments to clarify this point are in progress.

In other experiments, it was noted that while the catalytic activity was fairly stable, the inhibitory effect of ATP was modified by freezing or cold storage (4°C) of the preparation. For instance, although fresh preparations were inhibited 100% by ATP with NaF present (Table 1), after several days of freezing or refrigeration the PDC was less than 50% inhibited under these same conditions with either 1 mM ATP or 1 mM AMP-PNP. Additionally, with these frozen or refrigerated PDC preparations, this inhibitory effect was not blocked by added pyruvate and TPP or reversed by Mg^{++} addition. These experi-

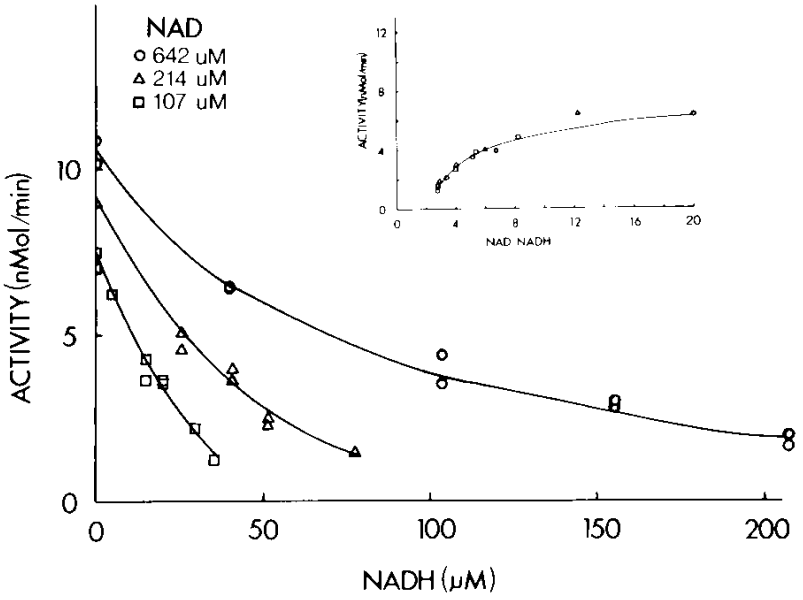

Fig. 4. The effects of NADH on PDC activity in the presence of different amounts of NAD. Activity was assayed as described in Materials and Methods at three indicated concentrations of NAD with varying amounts of NADH. Inset shows the data recalculated to show activity as related to the NAD/NADH ratio.

ments supported the suggestion that there was a labile protein kinase–phosphatase in this molluscan PDC and that ATP or AMP–PNP was exerting a direct inhibitory effect on the catalytic activity of the complex. These PDC preparations showing altered ATP or AMP–NADH sensitivity could be stored frozen for several weeks with little loss in overall catalytic activity (standard assay).

The NAD/NADH ratio in cells or mitochondria has been shown to have significant effects on the pyruvate dehydrogenase activities from mammals (Blass and Lewis, '73; Roche and Cate, '77; Wieland, '83) and *Ascaris* (Komuniecki et al., '79, '83). Although this effect may not be physiologically significant in mammals (Siess et al., '78), the NAD/NADH ratio may be very important in regulating anaerobic metabolism of the helminth parasites and molluscs (Saz, '81; Fields and Quinn, '81). The activity of the molluscan PDC was markedly affected by a change in the NAD/NADH ratio (Fig. 4). The greatest changes in the activity of the molluscan complex occurred at NAD/NADH ratios below 5 (high NADH levels) when the concentrations of NAD were 100, 200, 600 μM. In contrast, the *Ascaris* complex shows this sensitivity at a much lower NAD/NADH ratio (<1), which would allow the helminth PDC complex to function at higher NADH levels than the gill PDC reported here (see Komuniecki et al., '79).

The preliminary studies reported here indicate that PDC is present in bivalve tissue mitochondria and is similar to the mammalian and the *Ascaris* complexes in most respects. As with these PDCs, the ribbed mussel PDC is dependent on CoA, NAD, and pyruvate, and may be controlled by an ATP-dependent regulatory response characteristic of the protein phosphorylation/dephosphorylation scheme originally described for the mammalian PDCs (Linn et al., '69; Wieland and Jagow-Westermann, '69). There appears to be a secondary regulatory or inhibitory effect of ATP or ATP analogs on the catalytic activity of the gill PDC. Studies are currently underway to further characterize this PDC and determine its role in the regulation of alanine, pyruvate, and acetate metabolism in ribbed mussel gill tissue during osmotic stress and hypoxia.

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