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Kinetic and Regulatory Properties of Pyruvate Dehydrogenase from Ehrlich Ascites Tumor Cells[1]

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SUMMARY

Pyruvate dehydrogenase was partially purified from Ehrlich ascites tumor cell mitochondria and its kinetic properties were determined. The apparent K_m values for pyruvate, nicotinamide adenine dinucleotide, and coenzyme A (CoA) were 46 μM, 110 μM, and 36 μM, respectively. Reduced nicotinamide adenine dinucleotide and acetyl-CoA inhibited enzyme activity competitively to nicotinamide adenine dinucleotide (K_i = 22 μM) and CoA (K_i = 58 μM), respectively. Copurified α-ketoglutarate dehydrogenase displayed apparent K_m values for α-ketoglutarate, nicotinamide adenine dinucleotide, and CoA of 1.25 mM, 67 μM, and 50 μM, respectively.

Pyruvate dehydrogenase, but not α-ketoglutarate dehydrogenase, was inactivated specifically by adenosine triphosphate with concomitant phosphorylation, and it was reactivated at 10 mM Mg^{2+} by a protein fraction separated from the complex during purification. The rate of inactivation was decreased by pyruvate or pyrophosphate.

The existence of active and inactive forms of pyruvate dehydrogenase in Ehrlich ascites tumor cells was demonstrated. Active form and total activity were determined to be 74.0 ± 1.5 and 93.6 ± 4.9 munits/g packed cells (mean ± S.E., n = 25), respectively.

INTRODUCTION

Since the classical experiments of Warburg (22) and Cori and Cori (6), there have been several investigations of the enzymes of glycolysis and the tricarboxylic acid cycle in neoplastic tissues (for reviews see Refs. 11, 24, and 25). However, the enzymatic link between glycolysis and the Krebs cycle, namely, pyruvate dehydrogenase, has not been characterized so far in tumors. As Ehrlich ascites cells have been used frequently as a model for studies of pyruvate catabolism (2, 9, 17, 23, 24), it was the aim of the present study to determine the kinetic and regulatory properties of PDH[3] of this tumor.

MATERIALS AND METHODS

Materials

Acetyl-CoA, CoA, pyruvate, α-ketoglutaric acid, thiamine pyrophosphate, AMP, cAMP, ADP, ATP, ITP, GTP, CTP, UTP, dibutyryl-cAMP, NAD^+, NADH, and yeast RNA were obtained from Boehringer, Mannheim, Germany. Lubrol PX (M.G. 600) came from ICI, Frankfurt/Main, Germany. Polyethylene glycol (M.G. 6000), dithiothreitol, and 2-mercaptoethanol were products of Serva, Heidelberg, Germany. Protamine sulfate was produced by Schuchardt, Munich, Germany. [γ-^{32}P]ATP was purchased from the Radiochemical Centre, Amersham, England; PPO was from Merck, Darmstadt, Germany; and POPOP was from Packard Instrument GmbH, Frankfurt/Main, Germany. All other reagents were of analytic reagent grade. The donor mouse of Ehrlich ascites tumor cells was kindly supplied by Professor Rabes, Pathologisches Institut, the University of Munich. The tumor was propagated by i.p. injection of 0.4 to 0.5 ml of ascites fluid into NMRI mice and was harvested 8 to 11 days after transplantation.

Purification of PDH from Ascites Cells

All operations were performed at 2–4°. Ehrlich ascites cells were packed by low-speed centrifugation (1000 × g, 5 min) and were washed twice with a mixture of 0.075 M sucrose, 0.22 M mannitol, 1 mM triethanolamine, and 0.1 mM EDTA, at pH 7.2 (= isolation medium). The cells were ruptured by stirring for 10 min in a suspension of 3 volumes of 1 mM potassium phosphate buffer, pH 7.8. Then an equal volume of isolation medium with double the ionic strength was added. After stirring for 2 min the supernatant was collected after centrifugation for 10 min at 2000 × g. The sediment was resuspended and the procedure was repeated twice. Mitochondria in the pooled supernatants were collected by centrifugation for 15 min at 10,000 × g. After the mitochondria were washed with 20 mM potassium phosphate buffer, pH 7.0, until lactate dehydrogenase was removed, they were frozen and thawed twice, resuspended in a glass homogenizer with 2 volumes of 20 mM potassium phosphate buffer, pH 7.0, before the addition of an equal volume of 1% (w/v) Lubrol in the same buffer, and homogenized with a Teflon pestle. After centrifugation at 27,000 × g for 15 min, the supernatant was used for further purification by isoelectric precipitation and glycerol gradient centrifuga-

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[3] The abbreviations used are: PDH, pyruvate dehydrogenase complex (EC 1.2.4.1); cAMP, cyclic adenosine 3′:5′-monophosphate; dibutyryl-cAMP, N^6O^2-dibutyryl adenosine 3′:5′-monophosphate; PDH_a, dephospho form of pyruvate dehydrogenase.

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tion (28) or by protamine sulfate and polyethylene glycol fractionation (13). If necessary, enzyme solutions were concentrated by ultrafiltration with collodion bags SM 13200 (Sartorius Filter GmbH, Göttingen, Germany).

Assays for PDH

Depending on the stage of purification (see Tables 1 and 2), PDH activity was followed by 2 different methods as follows.

Assay A was performed according to the method of Scriba and Holzer (18) as modified by Wieland *et al.* (31). Arylamine acetyltransferase (EC 2.3.1.5) was prepared from pigeon liver (31). Assay B was carried out according to the method of Wieland *et al.* (30). The activity of α-ketoglutarate dehydrogenase was measured by Assay B, except that pyruvate was replaced by α-ketoglutarate.

One unit of enzyme activity corresponds to the formation of 1 μmole of acetyl-CoA (Assay A) or NADH (Assay B) per min at 25°. Product formation was linear with time and protein concentration. The specific activity is expressed as munits/mg protein. Protein was determined by the Biuret method (1) and corrected for turbidity according to the method of Keyser and Vaughn (10).

Determination of Active and Inactive Forms of PDH of Ascites Tumor Cells

Enzyme Extraction. Ascites fluid withdrawn from the peritoneal cavity of mice killed by cervical dislocation was injected immediately into liquid nitrogen. The cells were powdered in a precooled mortar under liquid nitrogen and were further disintegrated in a Minidismembrator (B. Braun, Melsungen, Germany) driven at full speed for 45 sec.

The following operations were carried out at 0–4°. One g of the powder was thawed and centrifuged for 2 min in a Model 3200 Eppendorf centrifuge. After the supernatant was collected, the precipitate was extracted with 0.5 ml of 20 mM K^+PO_4 buffer, pH 7.0, containing 1% Lubrol. The extraction procedure was repeated 3 times. (If Lubrol was omitted, only 20% of the PDH was extracted.) Prior to use in the enzyme assay, the pooled extracts were centrifuged for 5 min at 27,000 × *g*.

Assay of Active and Inactive Form of PDH. A mixture of 100 μl extract, 20 μl purified pig heart phosphatase (19), and 20 μl of 70 mM $MgCl_2$ was incubated at 25° and aliquots of 20 μl each were withdrawn at 0, 10, 20, and 30 min and were analyzed for PDH activity in Assay A. The PDH activity at 0 min represents the active portion (= PDH_a); maximum activity after incubation is regarded as total PDH activity. Usually, PDH activation was complete after 10 min. No activation occurred during the PDH assay or when Mg^{2+} was omitted from the mixture.

RESULTS AND DISCUSSION

Pyruvate dehydrogenase and α-ketoglutarate dehydrogenase were purified from Ehrlich ascites tumor cell mitochondria by methods originally developed for kidney (13), heart muscle (13, 28), and brain (20). Typical purification schemes are summarized in Tables 1 and 2. After glycerol gradient centrifugation (28), the ascites cell PDH was recovered in the same fraction numbers as the pig heart muscle enzyme, indicating that the sedimentation velocity

Table 1
Purification of PDH from 200 g of Ehrlich ascites tumor cells

Step	Total protein (mg)	Total activity (milliunits)	Specific activity	Recovery (%)
Mitochondrial fraction	1,469	10,091	6.9[a]	100
Lubrol extract	564	8,301	14.7[a]	83
Isoelectric precipitate (pH 5.6)	19.3	2,666	139[b]	26.7
Pooled glycerol gradient fractions	2.9	744	256[b]	7.4

[a] Activity determined by Assay A.
[b] Activity determined by Assay B.

Table 2
Purification of PDH and α-KGDH[a] from 250 g of Ehrlich ascites tumor cells

Step	Total volume (ml)	Total protein (mg)	Specific activities		Recovery (%)	
			PDH	α-KGDH	PDH	α-KGDH
Mitochondrial fraction	60	2,346	5.9[b]	39.9	100	100
Lubrol extract	165	1,459	7.5[b]	53.4	79	83
Protamine eluate	24.5	125	25.5[c]	92.3	23	12
After polyethylene glycol fractionation	0.7	13	231.3[c]		22	
	1.6	4.9		852.5		4

[a] α-KGDH, α-ketoglutarate dehydrogenase.
[b] Activity determined by Assay A.
[c] Activity determined by Assay B.

of the tumor PDH is comparable with that from nontumorous tissues (14, 20, 28).

Kinetic Properties of Purified PDH from Ehrlich Ascites Cells. Typical hyperbolic saturation curves were obtained for pyruvate, NAD^+, and CoA. The apparent K_m values as derived from Lineweaver-Burk plots are similar to those of PDH's from nontumorous tissues determined under the same conditions (see Table 3). No attempt was made to study the Mg^{2+} dependency, since the enzyme was unstable upon removal of Mg^{2+}. As may be seen from Table 4, appreciable enzyme activity was observed without added thiamine pyrophosphate, indicating that this cofactor was only partially removed from the enzyme during purification.

Acetyl-CoA and NADH were found to be inhibitors competitive to CoA and NAD^+, respectively. The inhibitor constants for NADH and acetyl-CoA were determined to be 22 and 58 μM, respectively. As may be seen from Table 3, similar K_i values for acetyl-CoA were found with PDH's from other animal sources.

On the basis of the K_i/K_m ratios for NADH/NAD^+ and acetyl-CoA/CoA of 0.2 and 1.6, respectively, it would appear that the overall activity of PDH of Ehrlich ascites tumor cells is more sensitive to changes of the intramitochondrial redox state than variations of the acetyl-CoA/CoA ratio, as has been proposed by Bremer (3) and Crompton and Laties (7) for the regulation of nontumorous PDH activity.

Kinetic Properties of α-Ketoglutarate Dehydrogenase Purified from Ehrlich Ascites Tumor Cells. The K_m values for α-ketoglutarate, NAD^+, and CoA as derived from Lineweaver-Burk plots were found to be 1.25 mM, 67 μM, and 50 μM, respectively. NADH inhibited the enzyme competitively to NAD^+, yielding a K_i value of 38 μM.

Inactivation of Purified PDH from Ehrlich Ascites Tumor Cells by ATP. Chart 1 shows that the purified ascites cell enzyme was inactivated with time when incubated together with ATP and Mg^{2+}. Whereas, with crude preparations, only partial inactivation was achieved (not shown), the purified enzyme was almost completely inactivated within 10 min at 0.1 mM ATP. The inactivation was strictly dependent on ATP. Other nucleotides such as GTP, ITP, UTP, CTP, and AMP, when tested in concentrations up to 0.5 mM, were ineffectual. Incubation with cAMP or dibutyryl-cAMP (up to 0.5 mM) did not affect enzyme activity nor did it change the rate of inactivation with ATP. The apparent K_m of PDH kinase for ATP determined according to the method previously described (20) was found to be 10 μM. Experiments with [γ-^{32}P]ATP indicate that the inactivation is accompanied by enzyme phosphorylation (Chart 2). In contrast to PDH, the activity of purified α-ketoglutarate dehydrogenase was not affected by ATP. From these results it seems reasonable to conclude that the purified ascites cell PDH contains an ATP-specific protein kinase, analogous to highly purified enzyme preparations from nontumorous tissues such as bovine kidney and heart (14, 15) and pork liver (15), heart (28), and brain (20).

Reactivation of Ascites PDH after Inactivation by ATP. As may be seen from Chart 3, the activity of phosphorylated PDH from ascites cells could be fully restored by incubation with high Mg^{2+} concentrations (10 mM) and a factor present in the top fractions of the glycerol gradient. The addition of Mg^{2+} alone did not yield any reactivation. By analogy with PDH interconversion systems studied in great detail (14, 15, 20, 28), we are inclined to call the gradient factor essential for reactivation of the ascites phospho-PDH "PDH-phosphatase." This is supported by the fact that highly purified pig heart PDH phosphatase replaced the ascites PDH phosphatase (Chart 3). On the other hand, ascites PDH phosphatase reactivated to some extent highly purified phospho-PDH from pig heart muscle (Chart 3). Thus it would appear that the phosphatase catalyzing PDH reactivation was removed from the complex by gradient centrifugation, in contrast to PDH kinase catalyzing PDH inactivation, as has been described already for PDH's from other sources (14, 15, 20, 28).

Protection against ATP Inhibition by Pyruvate. As may be seen from Chart 4, the inhibitory effect of a high concentration of ATP (0.5 mM) on PDH activity is prevented by pyruvate. Concentrations as low as 63 μM are effective. At 5 mM pyruvate, only a small inhibition, if any, occurs. Qualitatively, this is in accord with the results obtained with nontumorous PDH (14, 20, 29).

Effect of PP_i on Inactivation by ATP. PP_i inhibited the inactivation of PDH purified from Ehrlich ascites tumor cells competitively to ATP. The K_i value of 120 μM was 4 times

Table 4

Cofactor requirement of PDH and α-KGDH[a] purified from Ehrlich ascites tumor cells

Component omitted in Assay B	NADH formation by (ΔE_{334}/min)	
	PDH	α-KGDH
None	0.59	0.79
CoA	0	0
NAD^+	0	0
Mg^{2+}	0.115	0.24
Thiamine pyrophosphate	0.205	0.59

[a] α-KGDH, α-ketoglutarate dehydrogenase.

Table 3

Apparent K_m and K_i values (μM) of PDH's from various sources

Enzyme source	Pyruvate (K_m)	Co-A (K_m)	Acetyl-CoA (K_i)	NAD^+ (K_m)	Reference
Ehrlich ascites tumor	46	36	58	110	This paper
Pig heart muscle	28	5	29	40	30
Pig brain	45	9	10	80	20
Rat liver	30[a]	5	3	125[a]	32

[a] E. A. Siess, unpublished data.

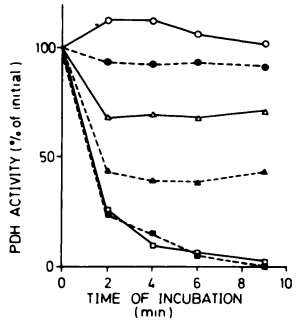

Chart 1. Inactivation of Ehrlich ascites tumor PDH by ATP. The reaction mixtures contained, in a total volume of 75 μl, 1.5 μmoles of potassium phosphate buffer, pH 7.0; 0.75 μmole of dithiothreitol; 75 nmoles of $MgCl_2$; various amounts of ATP to yield the final concentrations given below; and 21 μg of PDH protein. After incubation at 25° for the times indicated, 10-μl aliquots were analyzed for PDH activity (Assay B, see "Materials and Methods"); ○, no ATP; ●, 0.001 mM ATP; △, 0.005 mM ATP; ▲, 0.01 mM ATP; □, 0.1 mM ATP; ■, 1 mM ATP.

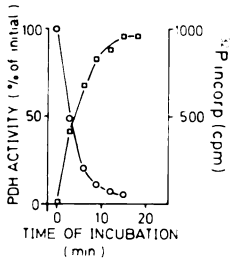

Chart 2. Concomitant phosphorylation during inactivation of purified Ehrlich ascites tumor PDH by [γ-^{32}P]ATP. The reaction mixture of 300 μl contained 6 μmoles of potassium phosphate buffer, pH 7.0; 30 nmoles of [γ-^{32}P]ATP (corresponding to 5 × 10^5 cpm); 30 nmoles of $MgCl_2$; and 12.4 milliunits of PDH. At the times indicated, 10-μl aliquots were tested for enzyme activity (○) (Assay B) and protein-bound radioactivity (□) according to the method of Mans and Novelli (16).

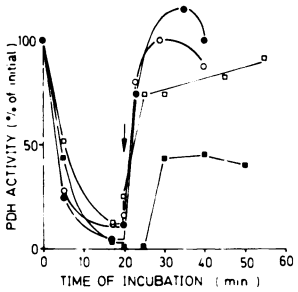

Chart 3. Interchangeability of Ehrlich ascites tumor PDH phosphatase with pig heart PDH phosphatase. The inactivation of PDH from Ehrlich ascites cells and pig heart muscle, respectively, was achieved as described in the legend to Chart 2. For reactivation (*arrow*), $MgCl_2$ concentration was increased to 10 mM and PDH phosphatase was added to yield the following enzyme combinations: ●, ascites tumor PDH (29 milliunits) and ascites tumor phosphatase (300 μg protein); ○, ascites tumor PDH (29 milliunits) and pig heart PDH phosphatase (191 μg protein); □, pig heart PDH (29 milliunits) and pig heart phosphatase (191 μg protein); ■, pig heart PDH (29 milliunits) and ascites tumor phosphatase (300 μg protein).

higher than that determined for PDH from pig heart muscle (33). Judged from the fact that K_i for PP_i is about 10 times higher than K_m for ATP, it seems unlikely that PP_i reaches concentrations in mitochondria sufficiently high to play a physiological role in the regulation of PDH interconversion. However, this question cannot be definitely answered until PP_i and ATP contents of ascites mitochondria are measured.

Occurrence of Active (Dephospho) and Inactive (Phospho) PDH in Ehrlich Ascites Tumor Cells. In order to measure the total PDH activity of a crude extract of Ehrlich ascites tumor cells, the inactive portion was converted to the active form. Thus, PDH activity obtained without additions represents the active form (PDH_a), whereas PDH activity of the extract fortified with Mg^{2+} and PDH phosphatase is regarded as total PDH. The validity of this procedure has been proven in previous experiments for the PDH system of the rat heart muscle (32). By this approach, PDH_a and total PDH activity of Ehrlich ascites tumor cells were determined to be 74.0 ± 1.5 and 93.6 ± 4.9 munits/g packed cells, respectively (mean ± S.E., n = 25).

According to Hepp *et al.* (8) about 30 nmoles of [1-^{14}C]pyruvate were decarboxylated per min per g of packed diploid Ehrlich ascites cells at 25°, and a similar value was reported by Katz *et al.* (9) for the hypodiploid strain. Rates of [1-^{14}C]lactate and [1-^{14}C]pyruvate oxidation of about 100 nmoles (23), 140 nmoles (9), 225 nmoles (26), and 320 nmoles (2), respectively, per g of packed cells per min at 25° have been reported for other strains of the Ehrlich ascites tumor, indicating that, with respect to PDH, strain variations similar to those for other enzyme activities may exist (12).

As to the rate-limiting step in glucose oxidation in Ehrlich ascites tumor cells, the relation among glycolytic pyruvate generation, PDH activity, and the capacity of the respiratory chain has to be considered. From a Q_{O_2} value for Ehrlich ascites tumor cells of about 8 (21, 27), one can estimate that PDH activity is commensurate with the rate of oxygen consumption. However, there is a striking discrepancy between glycolytic pyruvate production and PDH activity. From 1 to 1.8 μmoles of lactate aerobically produced per g of packed cells per min at 25° (4, 5), only some 5 to 15% could be catabolized by PDH at its full activity. A similar relationship has been found by Wenner and Weinhouse (27), who compared lactic acid and CO_2 formation from [*U*-^{14}C]glucose. Thus PDH activity appears to be a limiting factor in oxidative glucose breakdown by Ehrlich ascites tumor cells.

The fact that PDH exists in active and inactive forms in Ehrlich ascites tumor cells offers a possible explanation for the striking observations that [1-^{14}C]lactate oxidation was apparently not reduced by the addition of unlabeled pyruvate as expected from isotope dilution, in contrast to the effect of unlabeled lactate on [1-^{14}C]pyruvate decarboxylation (2). Since pyruvate, but not lactate, protects PDH from

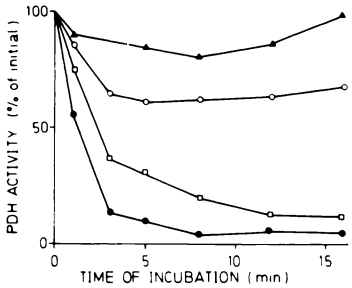

Chart 4. Protective effect of pyruvate against the inactivation of Ehrlich ascites tumor PDH by ATP. Reaction mixtures containing, in a total volume of 80 μl, 1.6 μmoles of potassium phosphate buffer, pH 7.0; 0.8 μmole of dithiothreitol; 40 nmoles of ATP; 100 nmoles of $MgCl_2$; and pyruvate to give the final concentrations stated below were incubated at 25°. At the times indicated, 10-μl aliquots were analyzed for PDH activity by Assay B. ●, no pyruvate; □, 62.5 μM pyruvate; ○, 2.5 mM pyruvate; ▲, 5 mM pyruvate.

inactivation by ATP, one can reason that because of unchanged PDH phosphatase activity, the level of PDH_a in the cells incubated with pyruvate is higher than in the cells incubated with lactate. This hypothesis is supported by the fact that the rate of [1-^{14}C]pyruvate oxidation (at 20 mM) is about twice that of [1-^{14}C]lactate (2). Thus by comparing the $^{14}CO_2$ formation from [1-^{14}C]lactate with that from [1-^{14}C]lactate, plus an equimolar amount of unlabeled pyruvate, the increase of PDH_a by a factor of 2 caused by pyruvate might compensate for the degree of isotope dilution of about the same magnitude. On the other hand, when $^{14}CO_2$ production from [1-^{14}C]pyruvate is related to that from [1-^{14}C]pyruvate plus an equivalent amount of unlabeled lactate, the isotope dilution becomes apparent, since in both cases decarboxylation rates are measured at the same PDH_a level.

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