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ISOLATED TUMORAL PYRUVATE DEHYDROGENASE CAN SYNTHESIZE ACETOIN WHICH INHIBITS PYRUVATE OXIDATION AS WELL AS OTHER ALDEHYDES

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ABSTRACT: Oxidation of 1 mM pyruvate by Ehrlich and AS30-D tumor mitochondria is inhibited by acetoin, an unusual and important metabolite of pyruvate utilization by cancer cells (1), by acetaldehyde, methylglyoxal and excess pyruvate. The respiratory inhibition is reversed by other substrates added to pyruvate and also by 0.5 mM ATP. Kinetic properties of pyruvate dehydrogenase complex isolated from these tumor mitochondria have been studied. This complex appears to be able to synthesize acetoin from acetaldehyde plus pyruvate and is competitively inhibited by acetoin. The role of a new regulatory pattern for tumoral pyruvate dehydrogenase is presented.

Several anomalies of the oxidative pathway for pyruvate by tumor mitochondria have been reported in the literature (2). Our recent finding was the discovery of the unusual metabolite, acetoin, as an important product of pyruvate utilization by Ehrlich ascites tumor mitochondria (preliminary results presented in 1). This product, which is formed by non-oxidative decarboxylation of pyruvate unique to tumors is not a normal metabolite. It has largely been found as a product of yeast (3) and bacterial metabolism (4). In tumor mitochondria, it is not only rapidly formed, but it is also very rapidly degraded in an ATP-dependent reaction. These processes do not occur in mitochondria from normal tissues. Also anomalous is the regulation of the tumoral pyruvate dehydrogenase complex: Lazo and Sols (5) reported that the PDH isolated from Ehrlich Lettré tumor mitochondria was activated by AMP which has no effect on the PDH of normal rat liver mitochondria. Moreover the inhibition of mitochondrial respiration in tumor cells by added non aliphatic aldehydes is also an anomalous feature (6-8). We report here that the in-vitro pathway for acetoin synthesis by tumor mitochondria requires pyruvate and "active" acetaldehyde as precursors. Moreover, acetoin is found to display inhibitory properties on pyru-

* Deceased.

ABBREVIATIONS: RLM: rat liver mitochondria; EATM: Ehrlich ascites tumor mitochondria; AS30-DTM: AS30-D tumor mitochondria; PDH: pyruvate dehydrogenase complex (pyruvate dehydrogenase [EC 1.2.4.1] + dihydrolipoamide transacetylase [EC 1.6.4.3] + dihydrolipoamide dehydrogenase [EC 1.6.4.3]; ALDH: aldehyde dehydrogenase; ATP: adenosine 5'-triphosphate; ADP: adenosine 5'-diphosphate; TPP: thiamine pyrophosphate; HE-TPP: hydroxyethyl-TPP; DNP: 2,4-dinitrophenol.

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vate oxidation by intact tumor mitochondria of two different cell lines and a competitive inhibition is produced by acetoin at the level of isolated PDH. Besides, a strong inhibition of mitochondrial respiration when pyruvate is substrate is observed when aliphatic aldehydes are present in the incubation medium, and especially with acetaldehyde, the necessary intermediate in the acetoin synthesis process. This observation suggests an important role acetoin could play for the survival of tumor cells by deviating the functioning of the tumoral PDH, which necessitates anyway the formation of the HE-TPP intermediate.

MATERIALS AND METHODS

1) Isolation procedures and assays. RLM were isolated according to (9) from 300 g adult male Sprague Dawley rats (Charles River, Willmington, MA). Mitochondria from Ehrlich and AS30-D tumor cells were prepared according to (10) from our cell lines maintained by culture at 37°C in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum, 10 mM glucose, 10 mM Hepes pH 7.4 and streptomycin (100 mcg/ml) + penicillin (100 U/ml). The cells were serially cultured in vitro 5 times before 5×10^6 cells/mouse (Ehrlich) or 200×10^6 cells/rat (AS30-D) were re-inoculated intraperitoneally for in vivo passage. PDH was isolated from these mitochondria according to (5) and its activity was assayed according to (11) by measuring initial rates of NADH appearance at 340 nm. Acetoin was determined colorimetrically as in (12).

2) Incubations.

* Mitochondrial respiration under state 4 conditions was carried out with a Clark oxygen electrode in a 1.5 ml cell containing the incubation medium [130 mM KCl, 10 mM MOPS, 2 mM phosphate], mitochondria (2 mg protein/ml). 1 mM pyruvate was added when needed. The incubation was performed at 30°C with the air bubbled medium under constant stirring. The state 3 systems contained in addition to the previous conditions 2 mM ADP.

* Incubations for acetoin formation by tumoral PDH were carried out in stoppered test tubes at 37°C for 120 min. The reaction medium contained: 0.1 M citrate buffer pH 6.0, 5 mM $MgCl_2$, 5 mM thiamine pyrophosphate and 0.4 mg PDH protein/ml. At time zero, substrate was added (10 mM pyruvate or 190 mM acetaldehyde, or both, or no substrate). At indicated times, 0.95 ml aliquots were taken, precipitated with 75 µl 70% ice-cold perchloric acid and neutralized with KOH. The precipitate was removed by 5 min centrifugation in an Eppendorf centrifuge and the clear supernatant immediately assayed for acetoin. Each sample was compared to a blank containing no proteins.

* Proteins were determined by the biuret method (13) using defatted bovine serum albumin (fraction V, Sigma) as a standard. Acetaldehyde, acetoin, methylglyoxal and thiamine pyrophosphate were purchased from Sigma Co.

RESULTS

Since we have shown the formation and utilization by EATM and by AS30-DTM of an unusual metabolite for mammalian cells, acetoin (1), it was important to study its fate. Fig. 1 shows that for a 0 to 2 mM acetoin concentration span, the ADP- or DNP- stimulated pyruvate oxidation rate by EATM was inhibited in a way directly proportional to acetoin concentration. Above 2 mM acetoin, the induced inhibition tended to reach a plateau (empty symbols); 5 mM acetoin was able to produce 50% inhibition of the stimulated respiratory rate (37 natom O/min/mg protein) (endogenous respiration was substracted). In contrast, a 0.5 mM ATP addition to the incubation medium was sufficient to totally prevent (or reverse if ATP was added after the acetoin addition) the acetoin-induced inhibition of pyruvate oxidation by EATM (full symbols). The same phenomena occurred to the same extent with mitochondria isolated from the AS30-D cells line (not shown). On the other hand, 1 mM acetoin (or more) could not inhibit the oxidation by EATM or by AS30-DTM of other substrate such as 1 mM glutamate, 1 mM α-ketoglutarate, 1 mM

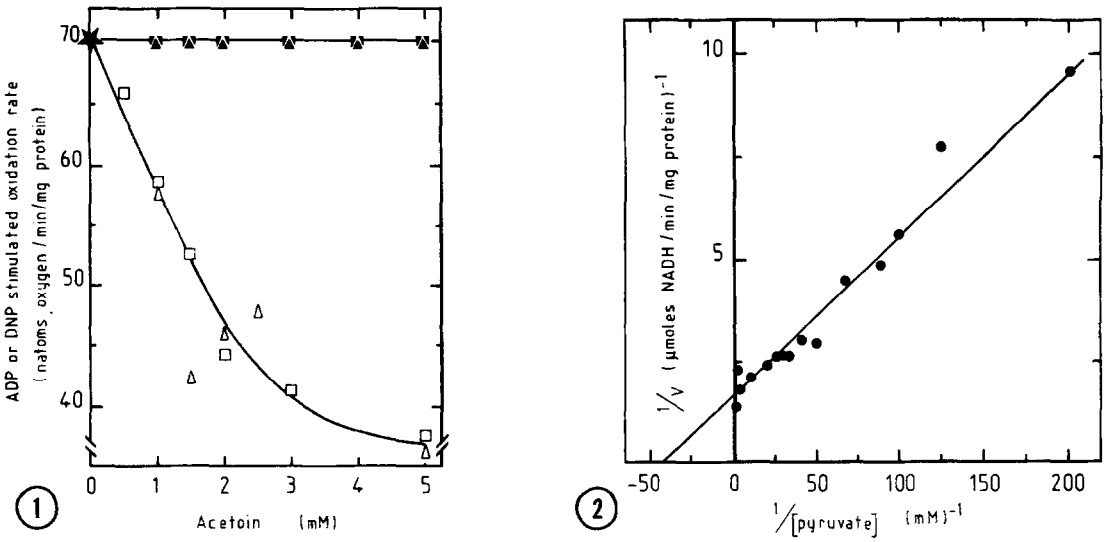


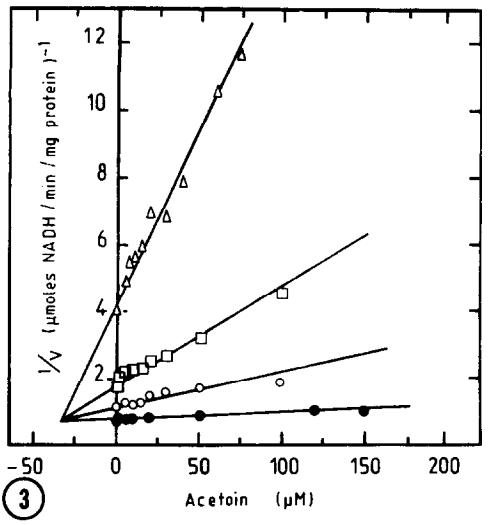
Fig. 1. Inhibition of ADP- or DNP-stimulated pyruvate oxidation rate in EATM by acetoin, and its prevention by ATP. EATM were prepared and incubated for 5 min as described under Materials and Methods. 1 mM pyruvate was the substrate. Stimulated respiration was induced either by 1.5 10^{-5} M DNP (Δ-Δ), or by 2 mM ADP for state 3 conditions (□-□), in the absence (open symbols) or the presence (full symbols) of 0.5 mM ATP. For the calculation, respiration due to endogenous substrates has been substracted. Results from 3 experiments.

Fig. 2. Lineweaver-Burk plot for the kinetic constants of the PDH isolated from EATM. PDH was isolated and assayed as described under Materials and Methods.

malate, or the oxidation of 1 mM pyruvate in the presence of any of these substrates (not shown). None of these observations could be done with RLM.

In order to explain these results one could incriminate either an inhibition of pyruvate transport or an inhibition of the oxidative decarboxylation of pyruvate by PDH, or both. Since pyruvate transport characteristics in EATM have been shown not to be different from the control ones (except for a slight Vmax increase) (14) we investigated a possible inhibition at the level of the tumoral PDH. Fig. 2 shows a Lineweaver-Burk plot for the kinetic constants of PDH from EATM. Cumulated K_m values for pyruvate were 26.5 ± 3.0 µM and 16.5 ± 2.1 µM for the PDH of EATM and AS30-DTM respectively (3 experiments). These values are 2 to 3.4 times lower than the K_m for pyruvate of the rat liver system (56 ± 4 µM). The Dixon plot (Fig. 3) shows acetoin as a typical competitive inhibitor with respect to pyruvate with a K_i of 36.3 ± 4.2 µM in EATM (3 experiments). The same kind of inhibition occurred with mitochondrial PDH from the AS30-D line with a K_i of 41.4 ± 2.1 µM (3 experiments).

As recently shown, the turnover for acetoin formation and utilization by tumoral mitochondria was too high to allow detection of any precursor under *in vivo* conditions. We therefore studied acetoin synthesis by the PDH isolated from EATM and AS30-DTM. Fig. 4 shows that PDH isolated from EATM was able to catalyze acetoin formation: with 10 mM pyruvate plus 190 mM acetaldehyde as



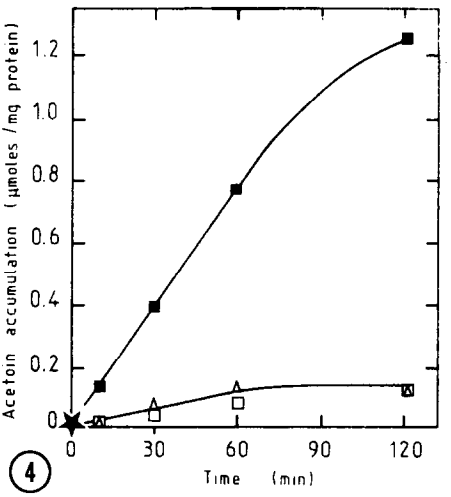


Fig. 3. Dixon plot for the competitive inhibition by acetoin of pyruvate tilization by PDH isolated from EATM. PDH from EATM was prepared and assayed as described under Materials and Methods. Pyruvate concentration studied were: 5 µM (Δ-Δ), 10 µM (□-□), 25 µM (○-○) and 1000 µM (●-●). 5 experiments were done, one typical experiment is presented.

Fig. 4. Acetoin formation by the PDH isolated from EATM. Incubations were carried out in stoppered test tubes at 37°C for 120 min. The reaction medium contained 0.1 M citrate buffer pH 6.0, 5.0 mM $MgCl_2$, 5.0 mM TPP and 0.4 mg protein/ml. At time zero, the substrate [either 190 mM acetaldehyde (Δ-Δ), or 10 mM pyruvate (□-□), or both (■-■)] was added and aliquots were taken, precipitated with perchloric acid, neutralized with KOH and centrifuged. The clear supernatant was immediately assayed for acetoin as described under Materials and Methods. 5 experiments were perfomed. One typical experiment is shown.

substrates (full symbols), the initial rate of synthesis was about ten times higher (14 nmol/min/mg protein) than with 10 mM pyruvate alone (1.3 nmol/min/mg protein) or with 190 mM acetaldehyde alone (0.84 nmol/min/mg protein), (empty symbols). The initial rate of acetoin formation was linear for at least a 60 min when acetaldehyde alone or pyruvate alone or both were substrates. PDH isolated from AS30-DTM was also able to catalyze acetoin formation with initial rates of 13, 12.3 and 20.6 nmol/min/mg protein when substrates were 10 mM pyruvate, 190 mM acetaldehyde, or both 10 mM pyruvate plus 190 mM acetaldehyde, respectively. In the absence of PDH or mitochondria no acetoin was formed in the presence of either pyruvate or acetaldehyde or both. Therefore, PDH appeared to be responsible for acetoin formation. Besides, acetaldehyde together with pyruvate appeared as the required intermediates for the *in vitro* synthesis of acetoin by tumoral PDH.

Acetaldehyde and other aliphatic aldehydes such as methylglyoxal, are normally oxidized by RLM and do not affect pyruvate decarboxylation by these mitochondria. In contrast tumor mitochondria from our Ehrlich and AS30-D lines could not oxidize these aldehydes (not shown). Moreover table I shows that these aldehydes are effective inhibitors of the DNP-activated respiration of EATM when 1 mM pyruvate was the substrate. We also found that excess substrate (10 mM pyru-

TABLE I. Inhibitory effects of some aldehydes and excess pyruvate on 1 mM pyruvate oxidation by Ehrlich tumor mitochondria

Additions	QO_2 (ng atom/min/mg protein)	
	- ATP	+ 0.5 mM ATP
None (DNP omitted)	20 ± 4	
None (DNP present)	73 ± 4	
Methylglyoxal (DNP present)	21 ± 4	35 ± 2
Acetaldehyde (DNP present)	32 ± 3	47 ± 2
Pyruvate (DNP present)	37 ± 5	73 ± 3

Mitochondria from Ehrlich tumor cells were prepared and incubated as described under Materials and Methods. Pyruvate (1 mM) was added as substrate. Maximum respiration rate was induced by the addition of $1.5\ 10^{-5}$ M DNP; no ADP was present. Various aldehydes or acetoin (each 1 mM) or excess pyruvate (10 mM) were added when indicated. When needed, 0.5 mM ATP was added before the substrate. Values are means ± S.E. of triplicate samples from 4 different experiments.

vate) was able to effectively inhibit by 51% the respiration rate of EATM. In every case studied, 0.5 mM ATP was able to reverse or prevent either partially (when 1 mM methylglyoxal or 1 mM acetaldehyde were tested) or totally (when 10 mM pyruvate was tested) the inhibition of mitochondrial respiration. Similar results were obtained when pyruvate oxidation was stimulated by 2 mM ADP. All of these results could be reproduced with AS30-DTM (not shown). Here too, and for every product tested, the inhibition of respiration on pyruvate could always be reversed or prevented if substrates such as glutamate, α-ketoglutarate or malate, (1 mM each) were added to pyruvate (not shown).

DISCUSSION

Even though our Ehrlich and AS30-D lines are highly glycolytic, we have found that isolated tumor mitochondria were readily able to oxidize pyruvate and support ADP- or DNP-stimulated respiration. Pyruvate supported respiration has previously been observed on erythropoietic tumor mitochondria (15). This was unexpected since usually oxidation of pyruvate to acetyl-CoA + CO_2 cannot continue for long without some means of deacylating the acetyl-CoA formed. We have previously shown that significant amounts of pyruvate were undergoing non-oxidative decarboxylation by tumor mirochondria and subsequent condensation to form acetoin (1). We proved through the present paper that acetoin could be formed through isolated PDH from acetaldehyde and pyruvate, as this occurs in yeast, but even at a slow rate from acetaldehyde alone, or from pyruvate alone, probably by condensation head to head of two acetaldehydes or of two decarboxylated pyruvates, one being under the "activated" form. However the presence of both acetaldehyde and pyruvate seems required for efficiency. Each reaction was necessarily catalyzed by PDH in the presence of TPP and Mg^{++} as cofactors. Because of the previously observed high turnover of acetoin in intact tumor mitochondria (1), we could not isolate any precursor of its synthesis, but on the light of the reactions observed *in vitro*, the same mechanism involving acetaldehyde may take place *in vivo*.

Veech et al. (16) reported that the PDH of alcoholic patients was able to catalyze the formation of acetoin from HE-TPP and acetaldehyde.

Interestingly, methylglyoxal has been reported to inhibit tumor cell division (17), aliphatic aldehydes react with thiol groups depressing protein synthesis (18) and causing membrane alterations (19). Other aldehydes induce inhibition of glycolysis and of mitochondrial respiration (6) and display antitumor activity (17,20,21).

In the present study the PDH inhibition by other aldehydes is very much in favor of free acetaldehyde intervention in the mechanism of acetoin formation in tumor tissues. Each aldehyde tested also inhibited the respiration of tumor mitochondria when 1 mM pyruvate was the substrate. The reversal or prevention of the inhibition due to the addition to pyruvate of other substrates (glutamate and malate that favor the entry of acetyl-CoA into the tricarboxylic cycle) suggest that the inhibition point is related to pyruvate oxidative decarboxylation through PDH. The most spectacular effect was due to ATP. The way this compound acts seems difficult to understand since ATP is a well known inhibitor of the decarboxylase moiety of the PDH.

The fact that mitochondria from cancer cell lines did not oxidize the aldehydes tested could be related to a main cytosolic location of AlDH: contrarily to normal liver where AlDH are primarily intramitochondrial and utilize aliphatic aldehydes, tumor AlDH isozymes are cytosolic and preferentially oxidize aromatic aldehydes using NADP as coenzyme (22). Alkonyi et al. (23,24) showed that acetaldehyde acted as an uncompetitive inhibitor affecting the function of the first subunit of pig heart mitochondrial PDH.

These observations shed some light on the anomalous regulatory characteristics of PDH from tumor mitochondria: this enzyme displays an unusual AMP-dependent activation (5), and we could determine acetoin as a competitive inhibitor with regards to pyruvate oxidative decarboxylation on the PDH. The K_i observed was higher (36.3 ± 4.2 µM) than the K_m for pyruvate (26.5 ± 3.0 µM) thus indicating a higher affinity for pyruvate than for acetoin on the PDH of EATM. The difference is strongly marked with PDH from AS30-DTM. The acetoin induced competitive inhibition on pyruvate oxidative decarboxylation by PDH explains its inhibitor effect on pyruvate oxidation by isolated intact tumor mitochondria; because of its neutral nature, acetoin readily passes through mitochondrial membrane and does not affect pyruvate transport into mitochondria. However the rate of acetoin disappearance in EATM being quite high (65 nmol/min/mg mitochondrial protein) (1), and because its competitive inhibition effect is not predominant over pyruvate decarboxylation it is more likely that it gets preferred access to its utilization by EATM. This is more evident with the AS30-D line since the K_i for acetoin is 3.4 times higher than the K_m for pyruvate at the level of the PDH.

In order to balance their Krebs cycle deficiency and keep up with ATP production these cells must increase their glycolysis rate. The pathway for acetoin formation (involving non-oxidative pyruvate decarboxylation) does not deviate the cell from this necessity but enters into the various biochemical adaptations to low oxygen tension environments cancer cells have acquired.

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