Biochimica et Biophysica Acta, 314 (1973) 1–7

BBA 46564

REGULATION OF PYRUVATE OXIDATION IN MITOCHONDRIA ISOLATED FROM FETAL AND ADULT RAT LIVER

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(Received February 28th, 1973)

SUMMARY

The effect of ATP on the velocity of oxygen uptake during the oxidation of pyruvate *plus* malate, in the presence of oligomycin, 2,4-dinitrophenol and fluorocitrate, was studied in mitochondria, isolated from the livers of adult and fetal rats.

It was found that the addition of ATP caused an inhibition in the rate of oxygen uptake of $21 \pm 6\%$ in mitochondria from adult rat liver and $49 \pm 8\%$ in mitochondria from fetal rat liver. Measurements of the velocity of oxygen uptake during the oxidation of pyruvate *plus* malate and of palmitoylcarnitine in adult rat liver mitochondria in the presence of ATP showed that the activity of pyruvate dehydrogenase was lower than the activity of citrate synthase.

In fetal mitochondria, addition of ATP resulted in an increase in the CoASH/acetyl-CoA ratio, indicating that pyruvate dehydrogenase was rate limiting here as well.

It is concluded that ATP inhibited pyruvate oxidation by phosphorylation of the pyruvate dehydrogenase complex, rather than by inhibiting citrate synthase under these conditions.

INTRODUCTION

Recently, Linn and co-workers[1,2] and Wieland and Siess[3] described the regulation of the activity of pyruvate dehydrogenase (EC 1.2.4.1) from various mammalian sources by phosphorylation and dephosphorylation. The enzyme is phosphorylated by an ATP-specific kinase, ADP being a competitive inhibitor; pyruvate protects the enzyme against phosphorylation[2]. The pyruvate dehydrogenase complex is inactive in the phosphorylated form. Activity is restored by the action of a Mg^{2+}-dependent phosphatase. By this combination of a kinase and a phosphatase the activity of the pyruvate dehydrogenase complex can be regulated by the ATP/ADP ratio of the medium.

Most of the studies concerning this type of regulation have been carried out on the isolated enzyme[1–3,9]. Some work has been done on the influence of the hormonal and nutritional status of the animal, on the activity of pyruvate dehydrogenase[4–8] and on the effect of adenine nucleotides on pyruvate oxidation in fat cell mitochondria[10].

The present study was undertaken to investigate the role of phosphorylation of pyruvate dehydrogenase as a regulatory mechanism in mitochondria isolated from fetal and adult rat liver in order to evaluate possible differences in the regulation of pyruvate oxidation in the two tissues. This is of special importance as the fetal hepatocyte produces more pyruvate and lactate by glycolysis than the mitochondrial system can oxidize[11] and the process of gluconeogenesis has not yet developed[12]. A preliminary report has been published[13].

MATERIALS AND METHODS

Rats of the T.N.O. (Duch Organization for Applied Science) strain were used. Mitochondria were isolated in 0.25 M sucrose according to the method of Hoogeboom and Schneider[14] from livers of adult and fetal (1–2 days before birth) rats. Incubations were carried out in an oxygraph or in a Dubnoff shaker. The basic reaction medium consisted of 10 mM K_2HPO_4; 150 mM KCl; 5 mM $MgCl_2$; 2 mM EDTA; 50 mM Tris–HCl; 50 μM 2,4-dinitrophenol; 100 μM fluorocitrate and 5 μg per ml oligomycin. Further additions and reaction conditions are given in the legends to the tables. Reactions were stopped by adding $HClO_4$, to a final concentration of 6%, to the medium which was then centrifuged. An aliquot of the supernatant was neutralized with KOH to pH 6–7 and, after standing for 1 h at 0 °C, was centrifuged. The supernatant was used for further analysis.

Intramitochondrial ATP and ADP were determined by separating the mitochondria from the reaction medium by rapid washing filtration, using the Millipore technique[15]. This procedure was immediately followed by extraction of the mitochondria with 2 ml 6% $HClO_4$. Pyruvate[16], malate[17], citrate[18], ATP[19] and ADP[20] were determined enzymatically by coupling to an NADH-dependent system.

A recycling system was used for the determination of CoASH and acetyl-CoA[21]. When small amounts of metabolites had to be determined (*e.g.* intramitochondrial ATP, ADP, CoA and acetyl-CoA), the change in absorption was measured either fluorometrically or with an Aminco-Chance dual-wavelength spectrophotometer. Protein was determined with the biuret method[22]. All reagents were of analytical grade. Fluorocitrate was purchased from K and K laboratories, oligomycin from Sigma and atractyloside was a gift from Professor V. Sprio to Professor E. C. Slater.

RESULTS

The activity of several enzymes of the Krebs cycle, *i.e.* citrate synthase[23] (EC 4.1.3.7) and isocitrate dehydrogenase[24] (EC 1.1.1.41) are influenced by ATP. Therefore it was necessary to use a reaction system which isolated the pyruvate dehydrogenase reaction as completely as possible. For this purpose the mitochondria were incubated with pyruvate, malate to generate a constant supply of oxaloacetate, fluorocitrate to inhibit further oxidation of citrate, oligomycin to inhibit ATPase and 2,4-dinitrophenol to stimulate respiration.

Under these conditions citrate synthase is part of the measuring system, when either the rate of oxygen uptake or pyruvate consumption and citrate accumulation are measured. If the activity of the pyruvate dehydrogenase complex alone is to

TABLE I

EFFECT OF THE INTRAMITOCHONDRIAL ATP/ADP RATIO ON CITRATE SYNTHESIS IN ADULT RAT LIVER MITOCHONDRIA

The incubation mixture contained basic medium, 6 mM malate, 6 mM pyruvate and 5.2 mg mitochondrial protein in a total volume of 2.7 ml. The mitochondria were preincubated for 2 min with ATP or ADP before the addition of malate and pyruvate. Temp. 25 °C, time 25 min. Values in μmoles, oxygen uptake in μatoms.

Addition (mM)		*Intramitochondrial ATP/ADP ratio*	*ΔPyr*	*ΔMal*	*ΔCit*	*ΔO*
ADP	*ATP*					
5	0.5	0.80	2.3	2.4	2.4	5.1
0.5	5	1.90	2.4	2.4	2.1	4.6

be estimated in this system, then it must be demonstrated that the activity of citrate synthase is not limiting the overall rate.

In Table I the results are given of a balance study of the oxidation of pyruvate *plus* malate. The disappearance of pyruvate and malate is calculated from the difference of two large numbers. The values are therefore subject to relatively large errors. Within experimental error, however, there was a 1:1 ratio between the disappearance of pyruvate and malate and the formation of citrate. Under these conditions no acetoacetate was formed. A 10% inhibition in the rate of oxygen consumption was observed in this case upon changing the ATP/ADP ratio to a higher value in adult rat liver mitochondria. An average inhibition of the rate of oxygen uptake of $21 \pm 6\%$ ($n=4$, range 10–30%) has been observed. This inhibition could be due to inhibition of either citrate synthase or pyruvate dehydrogenase. In order to distinguish between these possibilities the effect of different extramitochondrial concentrations of ATP on the rate of palmitoylcarnitine oxidation was measured. The results are shown in Table II. ATP, up to a concentration of at least 10 mM, had no effect on the oxidation of this substrate measured under the same conditions as

TABLE II

EFFECT OF THE EXTRAMITOCHONDRIAL ATP CONCENTRATION ON PALMITOYLCARNITINE OXIDATION IN ADULT RAT LIVER MITOCHONDRIA

The incubation mixture contained basic medium, 10 mM malate, 80 μM palmitoylcarnitine and 5.4 mg of mitochondrial protein in a total volume of 2.7 ml. Temp. 25 °C.

ATP (mM)	*Rate of oxygen consumption (natoms/min per mg protein)*
0	91
4	91
8	91
10	93

TABLE III

EFFECT OF ATP ON THE OXYGEN UPTAKE DURING THE OXIDATION OF PYRUVATE *PLUS* MALATE BY FETAL RAT LIVER MITOCHONDRIA

The incubation mixture contained basic medium, 10 mM malate, 10 mM pyruvate and 7.1 mg mitochondrial protein in a final volume of 2.7 ml. Temp. 25 °C.

Additions	*v (natoms O/min per mg protein)*
ADP, 10 mM	5.5
ATP, 10 mM	3.0

those of Table I. It should be stressed that in the presence of a high concentration of malate, rat liver mitochondria accumulate very little acetoacetate[25].

One can calculate therefore from Table II an activity for citrate synthase of about 30 nmoles/min per mg mitochondrial protein for adult rat liver mitochondria, even at the highest concentration of ATP added. From Table I the rate of pyruvate oxidation can be calculated to be 19–20 nmoles pyruvate/min per mg mitochondrial protein. Thus citrate synthase could not have been rate limiting during the oxidation of pyruvate *plus* malate in adult rat liver mitochondria. A similar conclusion was drawn by Schuster and Olson[26] for rabbit heart mitochondria.

The same experiments were performed with mitochondria isolated from fetal rat liver. In Table III the results of an experiment on the rate of oxygen uptake with pyruvate and malate as substrates are given. Addition of ATP instead of ADP results in a 45% inhibition in the rate of oxygen uptake. The average inhibition of the rate of oxygen consumption in fetal rat liver mitochondria was found to be $49 \pm 8\%$ ($n = 5$, range 40–58%). That citrate synthase is also not rate limiting in these mitochondria could be demonstrated by analyses of CoA and acetyl-CoA in the absence and presence of ATP (Table IV). An increase in the ratio CoASH/acetyl-CoA was observed upon addition of ATP, indicating that the formation of acetyl-CoA was inhibited rather than its removal by citrate synthase. It was necessary to use this indirect approach because it was found that fetal rat liver mitochondria exhibited a rate of fatty acid oxidation lower than that of pyruvate oxidation.

TABLE IV

EFFECT OF ATP ON THE CoASH/ACETYL-CoA RATIO IN FETAL RAT LIVER MITOCHONDRIA DURING THE OXIDATION OF PYRUVATE *PLUS* MALATE

The incubation mixture contained basic medium, 2 mM pyruvate, 2 mM malate and 1.3 mg mitochondrial protein in a final volume of 2.0 ml. Temp. 25 °C. Time 30 min. Values in nmoles (citrate) or nmoles/mg protein (CoASH and acetyl-CoA).

Additions	*ΔCitrate*	*CoASH*	*Acetyl-CoA*	*CoASH/acetyl-CoA*
None	98	3.1	0.8	3.9
ATP, 2.5 mM	70	2.5	0.3	8.3
ATP, 5.0 mM	58	3.0	0.3	10.0

TABLE V

EFFECT OF ATRACTYLOSIDE ON THE INHIBITION OF PYRUVATE OXIDATION BY ATP IN ADULT RAT LIVER MITOCHONDRIA

The incubation mixture contained basic medium, 10 mM malate, 10 mM pyruvate and 10.0 mg of mitochondrial protein in a final volume of 2.7 ml. Temp. 25 °C.

ATP (*mM*)	*Rate of oxygen uptake* (*natoms/min per mg protein*)	
	− Atractyloside	*+ Atractyloside (5 μg/ml)*
0	30.0	29.0
4	24.5	26.2
8	19.4	25.0

It can therefore be concluded that in intact adult and fetal rat liver mitochondria, ATP can inhibit the pyruvate dehydrogenase complex. The balance given in Table I demonstrates that besides an inhibition in the rate of oxygen uptake, a proportional decrease in citrate formation is observed. Such a decrease in the rate of citrate formation can also be demonstrated in fetal rat liver mitochondria as is illustrated in Table IV.

Under the conditions of these experiments accumulation of acetoacetate could not be measured. Accumulation of β-hydroxybutyrate cannot be expected because the presence of uncoupler maintains a highly oxidized state of the nicotinamide adenine dinucleotides. Furthermore, fetal liver mitochondria do not contain β-hydroxybutyrate dehydrogenase[27].

In the experiment of Table V the mitochondria were preincubated with atractyloside before the addition of ATP. In this way the entrance of adenine nucleotides into the mitochondria is inhibited. It can be seen from this experiment that under these conditions pyruvate oxidation is not as greatly affected by the extramitochondrial ATP as in the absence of atractyloside. It may therefore be concluded that the pyruvate dehydrogenase is localized on the inside of the atractyloside-sensitive barrier.

DISCUSSION

In the reaction system used, the activity of pyruvate dehydrogenase is rate limiting. The rate of oxidation of palmitoylcarnitine in adult liver mitochondria and the CoASH/acetyl–CoA ratios in the presence and absence of ATP in the case of fetal liver mitochondria demonstrate that citrate synthase cannot be rate limiting. A high intramitochondrial ATP concentration may stimulate the conversion of pyruvate to oxaloacetate which is catalyzed by pyruvate carboxylase (EC 6.4.1.1). In the case of adult rat liver mitochondria, less malate might then be necessary for the formation of citrate, which would result in a lowering of the oxygen consumed per citrate molecule formed. Table I illustrates, however, that ATP inhibits the oxygen consumption and citrate formation to the same extent. Furthermore, fetal rat liver mitochondria do not contain pyruvate carboxylase[28].

Table V illustrates that inhibition by ATP of pyruvate dehydrogenase is hardly demonstrable in the presence of atractyloside. The fact that a slight inhibition can still be observed may be due to an incomplete block of the adenine nucleotide translocator. The conclusion must be, that pyruvate dehydrogenase and its specific kinase are localized on the inside of the atractyloside-sensitive barrier. This finding implies that the activity of pyruvate dehydrogenase is affected by the intramitochondrial ATP/ADP ratio and only indirectly by the cytoplasmic ATP/ADP ratio.

For this reason the intramitochondrial ATP/ADP ratio has also been determined in adult rat liver mitochondria. Indeed, a correlation between the intramitochondrial ATP/ADP ratio and the activity of pyruvate dehydrogenase can be observed (Table I), inhibition of the enzyme occurring at a higher ATP/ADP ratio. The technique used for the determination of the intramitochondrial ATP/ADP ratio, *i.e.* the Millipore technique, has been subjected to criticism[29]. However, the presence of oligomycin excluded changes in this ratio by either oxidative phosphorylation or ATPase during the time taken to separate the mitochondria from the medium.

Adult rat liver mitochondria seem to be less sensitive to inhibition of pyruvate oxidation by ATP than fetal rat liver mitochondria (Tables I and III). Whereas with adult rat liver mitochondria an average inhibition of pyruvate oxidation of 21% could be observed, fetal rat liver mitochondria exhibited a much higher inhibition, up to 58% under the same experimental conditions. Rat liver mitochondrial pyruvate dehydrogenase is apparently less protected against ATP inhibition by pyruvate than the enzyme from pork liver, because Linn *et al.*[2] reported 60% protection by 0.5 mM pyruvate. This figure has been obtained on the isolated enzyme. It is not exactly known what the intramitochondrial pyruvate concentration is at an extramitochondrial concentration of 10 mM as used here in some experiments. This may in part explain the differences. Whether species differences contribute also to the different effect of pyruvate on the pyruvate dehydrogenase should be clarified by studies on the isolated enzyme.

The fact that inhibition can be observed in fetal rat liver mitochondria demonstrates that the kinase is already present at this stage of development. One explanation for the higher degree of inhibition in these mitochondria as compared to adult liver mitochondria may be a different ratio of the kinase to the phosphatase, the phosphatase in the fetal liver mitochondria being lower, resulting in a higher degree of phosphorylation of the pyruvate dehydrogenase.

A greater sensitivity to ATP inhibition of the fetal liver pyruvate dehydrogenase may play a role in the regulation of fatty acid synthesis. Fatty acid synthesis from glucose is high in fetal liver[30]. This pathway involves the pyruvate dehydrogenase. As further oxidation of acetyl-CoA by the Krebs cycle may be limited by succinate dehydrogenase activity in fetal rat liver mitochondria[31], fatty acid synthesis would continue unlimited unless the supply of carbon for fatty acid synthesis is regulated, for instance at the level of pyruvate dehydrogenase.

As with all studies with fetal rat liver, these experiments suffer from the disadvantage of being performed with material derived from a non-homogeneous tissue. However, Greengard *et al.*[32] have shown that at 1–2 days before birth 22% of the total volume of the fetal liver is occupied by hematopoietic cels. The results

of the present study are therefore relevant to fetal liver tissue because the contribution by the hematopoietic cells cannot be greater than 22%.

ACKNOWLEDGEMENT

These investigations were supported (in part) by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.). Thanks are due to Professor Dr J. M. Tager for valuable discussions.

REFERENCES

1 Linn, T. C., Pettit, P. H. and Reed, L. J. (1969) *Proc. Natl. Acad. Sci. U.S.* 62, 234–241
2 Linn, T. C., Pettit, P. H., Hucho, F. and Reed, L. J. (1969) *Proc. Natl. Acad. Sci. U.S.*, 64 227–234
3 Wieland, O. and Siess, E. (1970) *Proc. Natl. Acad. Sci. U.S.* 65, 947–952
4 Jungas, R. L. (1971) *Metabolism* 20, 43–63
5 Wieland, O., Siess, E., Schulze-Wethmar, F. H., v. Funcke, H. G. and Winter, B. (1971) *Arch. Biochem. Biophys.* 143, 593–601
6 Söling, H. D., Bernhard, G. and Janson, G. (1971) *FEBS Lett.* 13, 201–203
7 Wieland, O., v. Funcke, H. G. and Löffler, G. (1971) *FEBS Lett.* 15, 295–298
8 Weiss, L., Löffer, G., Schirman, A. and Wieland, O. (1971) *FEBS. Lett.* 15, 229–231
9 Siess, E., Wittman, J. and Wieland, O. (1971) *Hoppe Seyler's Z. Physiol. Chem.* 352, 447–452
10 Martin, B. R. and Denton, R. M. (1971) *Biochem. J.* 125, 105–112
11 Hommes, F. A., Kraan, G. P. B. and Berger, R. (1973) *Enzyme*, in the press
12 Ballard, F. J. (1971) *Biochem. J.* 124, 265–274
13 Berger, R. and Hommes, F. A. (1972) *Abstr. Commun. 8th Meet. Eur. Biochem. Soc., Amsterdam*, No. 1105
14 Hoogeboom, G. (1962) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. 1, pp. 16–19, Academic Press, New York
15 de Haan, E. J. and Tager, J. M. (1968) *Biochim. Biophys. Acta* 153, 98–108
16 Czok, R. and Lamprecht, W. (1970) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H. U., ed.), Vol. II, pp. 1407–1411, Verlag Chemie, Weinheim
17 Hohorst, H. J. (1970) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H. U., ed.), Vol. II, pp. 1544–1548, Verlag Chemie, Weinheim
18 Dagley, S. (1970) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H. U., ed.), Vol. II, pp. 1520–1523, Verlag Chemie, Weinheim
19 Lamprecht, W. and Trautschold, I. (1970) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H. U., ed.), Vol. II, pp. 2024–2032, Verlag Chemie, Weinheim
20 Jaworek, D., Gruber, W. and Bergmeyer, H. U. (1970) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H. U., ed.), Vol. II, pp. 2051–2055, Verlag Chemie, Weinheim
21 Michal, G. and Bergmeyer, H. U. (1970) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H. U., ed.), Vol. II, pp. 1900–1905, Verlag Chemie, Weinheim
22 Cleland, K. W. and Slater, E. C. (1953) *Biochem. J.* 53, 547–556
23 Srere, P. (1971) in *Advances in Enzyme Regulation* (Weber, G., ed.), p. 221, Pergamon Press, Oxford
24 Chen, R. F. and Plaut, G. W. E. (1963) *Biochemistry* 2, 1023–1027
25 Lopez-Cardoso, M. and van den Bergh, S. G. (1972) *Biochim. Biophys. Acta* 283, 1–15
26 Schuster, S. M. and Olsen, M. S. (1972) *J. Biol. Chem.* 247, 5088–5094
27 Hommes, F. A., Luit-de Haan, G. and Richters, A. R. (1971) *Biol. Neonat.* 17, 15–23
28 Ballard, F. J. and Hanson, R. W. (1967) *Biochem. J.* 104, 866
29 La Noue, K. F., Bryla, J. and Williamson, J. R. (1972) *J. Biol. Chem.* 247, 667–679
30 Ballard, F. J. and Hanson, R. W. (1967) *Biochem. J.* 102, 952–958
31 de Vos, M. A., Wilmink, C. W. and Hommes, F. A. (1968) *Biol. Neonat.* 13, 83–89
32 Greengard, O., Federman, M. and Knox, W. E. (1972) *J. Cell Biol.* 52, 261–272