THE ROLE OF THE TRICARBOXYLATE TRANSPORTING SYSTEM IN THE PRODUCTION OF PHOSPHOENOLPYRUVATE BY OX LIVER MITOCHONDRIA

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1. Introduction

Recent work from this laboratory has shown that the transport of phosphoenolpyruvate in mitochondria from rat liver, guinea-pig liver and rat heart can be mediated by the tricarboxylate transporting system [1]. In order to establish that this transporting system is responsible for the transport of phosphoenolpyruvate synthesised intramitochondrially to the cytosol (or surrounding medium), the production of phosphoenolpyruvate by ox liver mitochondria was investigated. The choice of ox liver was made since, as is the case for rabbit, chick and sheep liver, phosphoenolpyruvate carboxykinase, the enzyme responsible for phosphoenolpyruvate synthesis in gluconeogenesis, is largely mitochondrial [2, 3].

2. Methods

Fresh ox liver was obtained from the slaughter house and mitochondria were prepared on a small scale as described for rat liver mitochondria [4] in a medium containing 0.25 M sucrose, 0.4 mM EGTA, 5 mM Tris-Cl and 1% Bovine Serum Albumin, pH 7.4. The presence of albumin was found to be necessary in order that wellcoupled mitochondria were obtained. Pyruvate and phosphoenolpyruvate were estimated fluorimetrically by the method of Czok and Eckert [5]. Citrate was estimated by the method of Moellering and Gruber [6]. Benzene 123 tricarboxylate was obtained from the Aldrich Chemical Co., Milwaukie, Wisc.

Abbreviations:
FCCP: carbonyl-cyanide-*p*-trifluoro methoxy phenyl-hydrazone
PEP : phosphoenolpyruvate

3. Results

Ox liver mitochondria were found to possess similar characteristics to rat liver mitochondria in that exchanges of externally added phosphoenolpyruvate for intramitochondrial ^{14}C-L-malate and ^{14}-citrate could easily be demonstrated, these exchanges being inhibited by benzene 123 tricarboxylate in an analogous fashion to those in liver [1]. Rates of phosphoenolpyruvate/^{14}-citrate exchange were measured by the 'inhibitor stop' technique [1] and found to be of the order of 5 nmole/min/mg protein at 10°.

Ox liver mitochondria (300 mg protein) were incubated in 30 ml of a medium containing 125 mM KCl, 20 mM trischloride pH 7.4 with 5 mM 2-oxoglutarate, 2 mM inorganic phosphate and 1 μM FCCP at 22°. Under these conditions phosphoenolpyruvate was synthesised at a rate of 2–4 nmole/min/mg protein. After 5 min incubation 10 μM rotenone and 1 μg/ml antimycin were added when the mitochondria were separated from the surrounding medium by centrifugation and resuspended in a small volume of mitochondrial preparation medium. Aliquots (15 mg protein) of these mitochondria were then added to 1 ml incubation of a buffer containing 125 mM KCl 20 mM trischloride, pH 7.4 at 10° with the additions shown in table 1. After 2 min the mitochondria were separated by centrifugation at 0° at 18,000 *g* for 4 min and samples of the supernatant and pellet prepared for assay of pyruvate and phosphoenolpyru-

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Table 1
The distribution of phosphoenolpyruvate between the suspending medium and mitochondria in the presence of citrate, L-malate and benzene 123 tricarboxylate.

Additions	Supernatant (nmole)	Pellet (nmole)
None	57.3	83.1
Benzene 123 tricarboxylate (25 mM)	50.5	88.5
Citrate (1 mM)	139	4.6
Citrate (1 mM) + Benzene 123 tricarboxylate (25 mM)	62.3	74.6
L-Malate (1 mM)	115	24.6
L-Malate (1 mM) + Benzene 123 tricarboxylate (25 mM)	66.8	82.8
L-Malate (1 mM) + Pentylmalonate (25 mM)	111	29.9

See text for description of experiment. Pyruvate present was less than 5 nmole per incubation for both supernatant and pellet. Values given are the means of duplicate incubations.

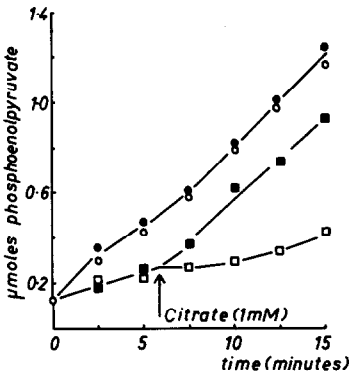

Fig. 1. Ox liver mitochondria (30 mg aliquots) were added to 10 ml of a medium containing 125 mM KCl, 20 mM trischloride, 5 mM 2-oxoglutarate, 2 mM inorganic phosphate and 1 μM FCCP pH 7.4 at 22°. The suspension was mechanically stirred and at the time intervals indicated 0.5 ml samples were removed into 0.25 ml 1.6 M $HClO_4$. The protein was separated off by centrifugation, the samples neutralised with 6 N triethanolamine/3 N potassium carbonate base and pyruvate and phosphoenolpyruvate were measured. (Irrespective of conditions the pyruvate appeared to be consistently one sixth of the value of PEP throughout the incubations.) Additions were none (o—o), benzene 123 tricarboxylate 20 mM (□—□), citrate 1 mM (•—•), benzene 123 tricarboxylate 20 mM with citrate (1 mM) added at 6.0 min (■—■).

vate described previously [7]. The presence of either citrate or L-malate caused a displacement of phosphoenolpyruvate from the mitochondria to the suspending medium this being blocked by benzene 123 tricarboxylate but not by pentylmalonate. Benzene 123 tricarboxylate added to control incubations showed more phosphoenolpyruvate associated with the mitochondrial pellet compared with controls indicating that some leakage of phosphoenolpyruvate had occurred from the mitochondria in the control incubations.

When ox liver mitochondria were incubated with 2-oxoglutarate, inorganic phosphate and uncoupling agent they produced phosphoenolpyruvate at an almost linear rate (fig. 1). This rate was inhibited 75% by the presence of 20 mM benzene 123 tricarboxylate. The addition of 1 mM citrate at 5 min to an incubation containing 20 mM benzene 123 tricarboxylate appeared to relieve the inhibition and the rate of phosphoenolpyruvate synthesis returned to normal. Addition of 1 mM citrate to the incubation in the absence of benzene 123 tricarboxylate did not cause an appreciable change in the rate of phosphoenolpyruvate synthesis in this experiment. However, in incubations of this type a stimulation by citrate was observed in five out of eight experiments, the maximum stimulation being a 98% increase in rate in one case. This variability of response to citrate may be due to the various amounts of endogenous citrate or L-malate present in the mitochondrial preparation. In the above experiment (fig. 1) 0.2 mM endogenous citrate remained unchanged throughout the incubation.

In an attempt to distinghuish between intra- and extra-mitochondrial pools of phosphoenolpyruvate present during incubations of this type, mitochondria were incubated for 5 min as described for fig. 1 with and without inorganic phosphate and then separated by centrifugation for 4 min at 0° at 18,000 *g*, supernatant and pellet extracts then being assayed for phosphoenolpyruvate. As expected, the supernatant values followed the same patterns as seen for fig. 1 (table 2) but the pellet analysis showed higher levels of phosphoenolpyruvate in the presence of benzene 123 tricarboxylate compared with controls, while in the presence of citrate, lower values than in controls were found. When both citrate and benzene 123 tricarboxylate were present values were lower than with benzene

Table 2
The distribution of pyruvate and phosphoenolpyruvate between mitochondria and suspending medium after incubation with substrate for 5 min.

Substrate	Additions	Supernatant		Pellet	
		Pyruvate	Phosphoenol-pyruvate	Pyruvate	Phosphoenol-pyruvate
		(nmoles)			
2-Oxoglutarate (5 mM) FCCP (1 μM)	None	35.0	206	10.8	41.2
2-Oxoglutarate (5 mM) FCCP (1 μM)	Benzene 123 Tricarboxylate (20 mM)	11.4	82.9	15.4	62.6
2-Oxoglutarate (5 mM) FCCP (1 μM)	Citrate (1 mM)	32.8	206	5.3	22.5
2-Oxoglutarate (5 mM) FCCP (1 μM)	Citrate (1 mM) + Benzene 123 Tricarboxylate (20 mM)	21.6	128	5.6	31.3
2-Oxoglutarate (5 mM) FCCP (1 μM) Phosphate (2 mM)	None	32.2	194	7.4	31.4
2-Oxoglutarate (5 mM) FCCP (1 μM) Phosphate (2 mM)	Benzene 123 Tricarboxylate (20 mM)	12.9	86.6	11.9	44.3
2-Oxoglutarate (5 mM) FCCP (1 μM) Phosphate (2 mM)	Citrate (1 mM)	41.7	246	4.9	20.1
2-Oxoglutarate (5 mM) FCCP (1 μM) Phosphate (2 mM)	Citrate (1 mM) + Benzene 123 Tricarboxylate (20 mM)	29.1	174	8.1	32.2

15 mg aliquots of ox liver mitochondria were added to 5 ml incubations of a buffer containing 125 mM KCl, 20 mM trischloride pH 7.4 at 22° with the substrates and additions present as indicated. See text for further details.

123 tricarboxylate alone. These effects occurred in incubations both with and without phosphate and were highly reproducible. The addition of rotenone (1 μM) and antimycin (0.5 μg/ml) to the incubation prior to separation had little effect on the observed pattern of distribution. The smaller amounts of pyruvate which were measured appeared to bear a constant ratio to the amount of phosphoenolpyruvate present. Whether this pyruvate was derived from phosphoenolpyruvate breakdown through pyruvate kinase or from decarboxylate of oxaloacetate could not be ascertained.

Lastly, when mitochondria were incubated with 2-oxoglutarate, inorganic phosphate and uncoupling agent in the presence of increasing concentration of benzene 123 tricarboxylate it was found that half maximal inhibition of phosphoenolpyruvate production occurred at 8 mM inhibitor. Obviously complete inhibition cannot be observed in this system because up to 4 nmole/mg of protein may be intramitochondrial and total endogenous phosphoenolpyruvate before incubation may be up to 6 nmole/mg of protein (fig. 2).

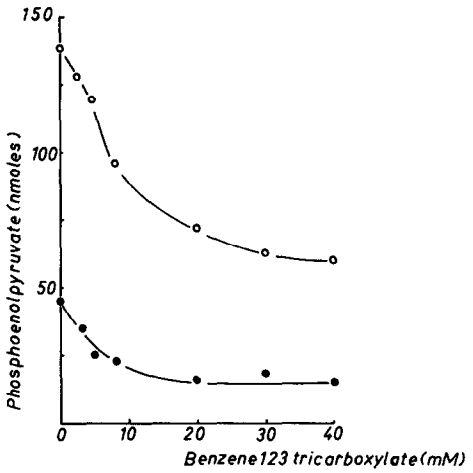

Fig. 2. Ox liver mitochondria (8 mg aliquots) were incubated as described for fig. 1 for 6 min at 22° in the presence of increasing concentrations of benzene 123 tricarboxylate. The reaction was stopped by addition of $HClO_4$ and extracts were assayed for oyruvate (●—●) and phosphoenolpyruvate (○—○).

4. Discussion

Ox liver mitochondria, which show the same anion exchange characteristics as rat liver mitochondria with respect to phosphoenolpyruvate are more suitable for the investigation of phosphoenolpyruvate transport because they have the ability to synthesise this compound intramitochondrially. That the citrate transporting system can exchange intramitochondrial phosphoenolpyruvate for externally added L-malate or citrate is indicated by the fact that displacement of phosphoenolpyruvate from the pellet to the supernatant at 10° is brought about by the additions of these ions, these diplacements being blocked by benzene 123 tricarboxylate and not 2-pentylmalonate [1, 8]. The displacement of phosphoenolpyruvate by citrate had already been shown by Gamble and Mazur [9] in rabbit liver mitochondria.

In a situation where mitochondria are producing phosphoenolpyruvate at a constant rate it seems that the requirement for an added counter-ion for exit of phosphoenolpyruvate from the mitochondria is not always evident. However, the fact that benzene 123 tricarboxylate, an inhibitor of citrate transport, markedly slows down the rate of phosphoenolpyruvate production does implicate a role for the tricarboxylate transporting system in phosphoenolpyruvate production, especially when it is observed that small amounts of added citrate appear to reverse the inhibition. L-Malate was also shown to relieve the inhibition by benzene 123 tricarboxylate but the possibility that it might act through providing further oxaloacetate for phosphoenolpyruvate synthesis could not be discounted. The higher mitochondrial levels of phosphoenolpyruvate in the presence of benzene 123 tricarboxylate compared with controls as well as the lower levels in the presence of citrate further indicate that the site of inhibition of phosphoenolpyruvate production by benzene 123 tricarboxylate is at the level of the membrane. Presumably the increase in phosphoenolpyruvate concentration intramitochondrially in the presence of this inhibitor slows down further synthesis by product inhibition of phosphoenolpyruvate carboxykinase, so that the total phosphoenolpyruvate synthesised in a given period of time is less when the exit of this compound from the mitochondrial compartment is prevented. The concentration of benzene 123 tricarboxylate (8 mM) required to produce half-maximal inhibition of phosphoenolpyruvate production may appear to be rather high especially when one considers that it is competing only with endogenous citrate and L-malate for the transport site and that its K_i is 0.16 mM as measured by inhibition of tricarboxylate oxidation. However, in a dynamic system such as this, wherever a competitive inhibitor for a reaction is present, its effect may often be diminished by a rise in the concentration of the substrate for that reaction. In the case of phosphoenolpyruvate production, the inhibition of transport by lower concentrations of benzene 123 tricarboxylate may be overcome by a rise in phosphoenolpyruvate concentration. This hypothesis fits with the data obtained from separation experiments but it should be emphasised here that the data from such experiments must be interpreted with caution since it is possible that metabolite levels may alter significantly during the separation process, though in this case the addition of rotenone and antimycin to prevent further oxidative metabolism made no difference to the results. Taking these difficulties into consideration the results of these investigations are highly suggestive of a major role for tricarboxylate transporting system in the transport of phosphoenolpyruvate from its site of synthesis within the mitochondrial matrix to the cytoplasm.

Whether any control on the rate of gluconeogenesis could be exerted at this step *in vivo* remains an open question, though the author would agree with previous investigators [9–13] that phosphoenolpyruvate synthesised intramitochondrially has rapid access to the suspending medium in most *in vitro* circumstances so far tested.

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