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Competition for energy between phosphoenolpyruvate and citrulline synthesis in guinea-pig liver mitochondria

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SUMMARY

1. The interrelationship between citrulline synthesis and phosphoenolpyruvate formation has been studied in guinea-pig liver mitochondria incubated with glutamate in State 3 and in the presence of uncoupler and oligomycin.

2. In coupled mitochondria the rate of phosphoenolpyruvate production was limited by a higher capacity of aspartate aminotransferase than that of phosphopyruvate carboxylase for the intramitochondrial oxalacetate. Citrulline formation was low due to the small production of NH_3 since glutamate oxidation in State 3 proceeds *via* the transamination pathway.

3. Inhibition of aspartate aminotransferase by aminooxyacetate in State 3 resulted in increases in both phosphoenolpyruvate and citrulline synthesis. Under uncoupled conditions, however, an increase of phosphoenolpyruvate formation was accompanied by a decrease of both citrulline production and the ATP content of the incubation medium. Restoration of the citrulline production was observed on the addition of exogenous ATP.

4. The results indicate that when energy is generated *via* substrate-level phosphorylation, the inhibition of citrulline production is probably due to a higher availability of GTP to the phosphopyruvate carboxylase than to the nucleoside diphosphate kinase.

The urea cycle and gluconeogenesis are among those metabolic pathways requiring the participation of both mitochondrial and cytoplasmic enzymes. It is generally accepted that in mammalian liver the first two steps of the urea cycle *i.e.* the formation of carbamyl

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Abbreviations: *P*-enolpyruvate, phosphoenolpyruvate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

phosphate and the carbamylation of ornithine to citrulline take place in the mitochondria[1,2] whereas enzymes involved in the other steps are localized in the cytosol[3]. The formation of citrulline in rat-liver mitochondria has been extensively studied in Slater's laboratory[4–8]. However, rat-liver mitochondria differ significantly from several other animal species with respect to the subcellular distribution of the phosphopyruvate carboxylase [GTP–oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1. 1.32], a key enzyme of the gluconeogenic pathway. In the rat, most of the activity is found in the cytosol[9] whereas pigeon[10] and chicken[11] produce phosphoenolpyruvate intramitochondrially. On the other hand, in guinea pig[9], rabbit[9] and human[12] phosphopyruvate carboxylase is present both in the cytosol and in the mitochondria.

Since both citrulline and *P*-enolpyruvate synthesis require provision of energy it seemed interesting to study the interrelationship between these two processes in the same cellular compartment. Experiments presented in this paper were carried out with guinea-pig liver mitochondria prepared by a minor modification of the method of Schneider and Hogeboom[13]. The isolation medium contained 225 mM mannitol, 75 mM sucrose and 0.1 mM EDTA.

The reaction mixture contained 15 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 40 mM Tris–HCl buffer, 10 mM ornithine, 30 mM $KHCO_3$, 20 mM potassium phosphate buffer, 10 mM glutamate, about 3–4 mg mitochondrial protein/ml and the additions indicated in the legends to the tables and figures. The final pH was 7.4. In order to vary the intramitrochondrial ATP level, mitochondria were incubated under different respiratory states produced by the following additions to the basic medium: State 3, 30 mM glucose, 0.1 mM ADP and hexokinase (2–5 units/ml); Uncoupled + oligomycin, 0.2–0.5 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and oligomycin (1 μg/mg protein). The reaction was carried out in a chamber maintained at 30 °C as described previously[14]. For measurements of the total content of metabolites in the reaction medium, 2-ml aliquots were removed at either 5-min intervals up to 20 min (Tables I and II) or 2-min intervals up to 8 min (Figs. 1 and 2) and added to tubes containing 0.2 ml 35% $HClO_4$. The $HClO_4$ in the supernatant after centrifugation was precipitated as $KClO_4$ in the cold.

The determination of *P*-enolpyruvate, aspartate and glucose 6-phosphate was done spectrophotometrically while ATP was measured fluorometrically by the method of Williamson and Corkey[15]. Pyruvate kinase (EC 2.7.1.40), aspartate aminotransferase (EC 2.6.1.1), malate dehydrogenase (EC 1.1.1.37) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) used for assays of metabolites were isolated and purified according to Tietz and Ochoa[16], Sizer and Jenkins[17], Englard and Siegel[18] and Kuby and Noltmann[19], respectively. $(NH_4)_2SO_4$-free hexokinase (EC 2.7.1.1) was purchased from Koch and Light while lactate dehydrogenase (EC 1.1.1.27) was provided by Polskie Odczynniki Chemiczne (Poland). Citrulline was determined by the method of Archibald[20] as described by Charles *et al.*[5]. Mitochondrial protein was measured by the biuret method according to Cleland and Slater[21].

Since amino acids are substrates of both the gluconeogenic pathway and urea production, glutamate was chosen as the oxaloacetate precursor and NH_3 donor for *P*-enol-

TABLE I

EFFECT OF AMINOOXYACETATE AND NH_3 ON THE FORMATION OF *P*-ENOLPYRUVATE, CITRULLINE, ASPARTATE AND GLUCOSE 6-PHOSPHATE IN MITOCHONDRIA INCUBATED IN STATE 3

The reaction mixture contained the basic components *plus* 0.1 mM aminooxyacetate and 10 mM NH_4Cl where indicated. Values shown are means ± S.E. of mean of 4 separate experiments and the units are nmoles produced/min per mg protein.

Additions	Δ *P-enolpyruvate*	Δ *Citrulline*	Δ *Aspartate*	Δ *Glucose 6-phosphate*
None	0.5 ± 0.1	1.5 ± 0.3	10.9 ± 0.6	76 ± 2
Aminooxyacetate	2.4 ± 0.3	2.3 ± 0.2	0	70 ± 2
NH_4Cl	0.4 ± 0.1	6.0 ± 1.0	10.0 ± 0.7	61 ± 3
Aminooxyacetate + NH_4Cl	2.7 ± 0.4	5.2 ± 1.4	0	54 ± 8

TABLE II

EFFECT OF AMINOOXYACETATE, NH_3 AND EXOGENOUS ATP ON THE FORMATION OF *P*-ENOLPYRUVATE, CITRULLINE, ASPARTATE AND ATP IN MITOCHONDRIA INCUBATED IN THE PRESENCE OF FCCP AND OLIGOMYCIN

The reaction mixture contained the basic components *plus* 0.1 mM aminooxyacetate, 10 mM NH_4Cl, 5 mM ATP and 0.5 μM rotenone where indicated. Values shown are means ± S.E. of mean of 3 experiments and the units are nmoles produced/min per mg protein.

Additions	Δ *P-enolpyruvate*	Δ *Citrulline*	Δ *Aspartate*	*ATP levels*
None	4.3 ± 0.9	2.9 ± 0.3	9.1 ± 0.5	5.3 ± 1.0
Aminooxyacetate	10.1 ± 1.1	0.9 ± 0.1	0	2.5 ± 0.2
NH_4Cl	3.1 ± 0.7	2.8 ± 0.1	9.3 ± 1.1	3.9 ± 0.1
Aminooxyacetate + NH_4Cl	8.9 ± 1.4	0.9 ± 0.1	0	2.3 ± 0.3
ATP	2.3 ± 0.4	3.1 ± 0.4	9.0 ± 0.8	–
ATP + aminooxyacetate	8.4 ± 1.1	2.8 ± 0.2	0	–
ATP + NH_4Cl	2.9 ± 0.9	7.1 ± 0.9	9.6 ± 0.8	–
ATP + aminooxyacetate + NH_4Cl	6.1 ± 1.4	6.5 ± 1.0	0	–
ATP + NH_4Cl + rotenone	–	16.2 ± 2.2	–	–

pyruvate and citrulline synthesis, respectively. Moreover, oxidation of glutamate in the tricarboxylic acid cycle is able to provide energy required for both processes.

Table I shows the rates of both *P*-enolpyruvate and citrulline synthesis in mitochondria incubated with glutamate in State 3. In agreement with previous observations (Bryla, J., Fukami, M.H. and Williamson, J.R., unpublished), very little *P*-enolpyruvate formation occurred (0.5 nmole/min per mg protein) since under these conditions the glutamate transamination pathway[22] competes efficiently with phosphopyruvate carboxylase for the intramitochondrial oxaloacetate. Addition of aminooxyacetate, an inhibitor of aminotransferase[23], resulted in about 5-fold stimulation of the *P*-enolpyruvate production. On the other hand, the rate of citrulline synthesis was only slightly increased in the presence of aminooxyacetate, although the aspartate aminotransferase was completely

inhibited (no aspartate formation) and energy production was not decreased as judged from the glucose 6-phosphate generation. When NH_4Cl was included in the reaction medium, the rate of citrulline synthesis was stimulated up to 6 nmoles/min per mg protein, indicating that NH_3 production is a limiting factor for the formation of citrulline under State 3 conditions. Stimulation of citrulline synthesis in the presence of exogenous NH_3 was accompanied by a significant decrease of the glucose 6-phosphate formation.

In order to decrease the energy production, mitochondria were incubated in the presence of oligomycin and FCCP. Under these conditions GTP is generated *via* the substrate-level phosphorylation and ATP is produced from GTP *via* nucleoside diphosphate kinase (EC 2.7.4.6). Oligomycin was added in order to inhibit dissipation of ATP by the mitochondrial ATPase. Although uncoupler is known to switch the glutamate transamination to dehydrogenation[22], under the conditions of our experiments, the rates of aspartate formation were only 10–20% lower than measured in State 3. Although it has been proposed that the transport of aspartate is energy dependent[24], efflux of aspartate under uncoupled conditions could presumably proceed, since ATP levels in the reaction medium containing FCCP + oligomycin were not lower than 2.3 nmoles/mg protein.

In the presence of uncoupler and oligomycin, glutamate oxidation was 50–80% greater than that measured in State 3. Since aspartate production was slightly decreased, this indicates that under uncoupled conditions glutamate is oxidized *via* both the transamination and the dehydrogenation pathways resulting in an increase of *P*-enolpyruvate synthesis to 4.3 nmoles/min per mg protein. This stimulation could also be due to a higher intramitochondrial oxaloacetate concentration available for phosphopyruvate carboxylase since in the uncoupled state the NADH level is lower than that in State 3. The addition of aminooxyacetate to the uncoupled mitochondria increased *P*-enolpyruvate formation 2–3-fold, indicating that inhibition of the glutamate transamination makes more oxaloacetate available for the phosphopyruvate carboxylase. Our studies support the suggestion that a physiological uncoupling process is necessary for mitochondrial *P*-enolpyruvate formation during gluconeogenesis[25,26]. However, there was no evidence that the energy requirement for the mitochondrial *P*-enolpyruvate production could be met by transphosphorylation from the ATP pool[27,28]. On the contrary, addition of exogenous ATP to uncoupled mitochondria resulted in a marked decrease of *P*-enolpyruvate formation.

When no additions were made to the uncoupled mitochondria, citrulline synthesis was approximately double (2.9 nmoles/min per mg protein) that in State 3, presumably due to an increased NH_3 production during glutamate deamination. However, when aminooxyacetate was added to inhibit aspartate aminotransferase, the rate of citrulline production decreased to 0.9 nmole/min per mg protein, even in the presence of NH_4Cl. Since under these conditions ATP levels were also decreased (5.3 and 2.5 nmoles/mg protein in the absence and in the presence of aminooxyacetate, respectively), it seems that the inhibition of citrulline synthesis on the addition of aminooxyacetate was due to a decreased generation of ATP *via* the nucleoside diphosphate kinase. This conclusion is also supported by the following observations: (i) no inhibition of citrulline production occurred when aminooxyacetate was added in the presence of exogenous ATP; (ii) the K_m for ATP

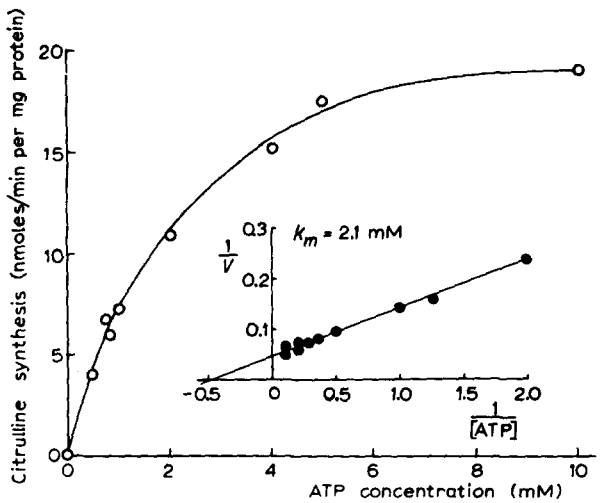

Fig. 1. Effect of ATP concentration on the rate of citrulline formation in mitochondria incubated in the presence of FCCP and oligomycin. Experimental conditions as in Table II with the exception that glutamate was omitted, and 0.5 μM rotenone, 10 mM NH_4Cl and ATP at the indicated concentrations were added to the reaction mixture. Samples were taken at 2, 4, 6 and 8 min to establish rates of citrulline formation. Values shown are means of two experiments.

was found to be 2.1 mM (Fig. 1) *i.e.* in the range of concentrations found in the mitochondrial mixture in the presence of aminooxyacetate. Since ATP is distributed between the mitochondria and the incubation medium[14,29], the intramitochondrial ATP content is presumably below 2 nmoles/mg protein, so it might limit the citrulline formation.

Addition of NH_4Cl to the mitochondria incubated with glutamate, FCCP and oligomycin did not affect the citrulline synthesis, indicating that NH_3 production in the uncoupled mitochondria is not rate limiting unless an exogenous source of energy is present. NH_4Cl in the presence of ATP stimulated citrulline formation 2–3-fold (7.1 and 6.5 nmoles/min per mg protein without and with aminooxyacetate, respectively). The K_m for NH_3 was found to be about 0.5 mM (Fig. 2). Assuming that NH_3 is easily permeable through the mitochondrial membrane[30], one can calculate that the concentration of this compound would be not higher than 0.2–0.4 mM after incubation of the uncoupled mitochondria with glutamate for 20 min. Thus, NH_3 production might limit the rate of citrulline synthesis under conditions of high energy generation.

In agreement with Graafmans *et al.*[1], uncoupled mitochondria produced the highest amounts of citrulline when oxidation of the substrate was inhibited by rotenone, and ATP and NH_4Cl were used as energy source and NH_3 donor, respectively. These data do not confirm the suggestion that respiratory inhibitors and uncoupling agents inhibit the entry of ornithine into the mitochondria, made on the basis of studies of the effect of ornithine on the mitochondrial swelling under various conditions[31].

The results presented in this paper indicate that in guinea-pig liver mitochondria, high rates of both *P*-enolpyruvate and citrulline synthesis are maintained when:

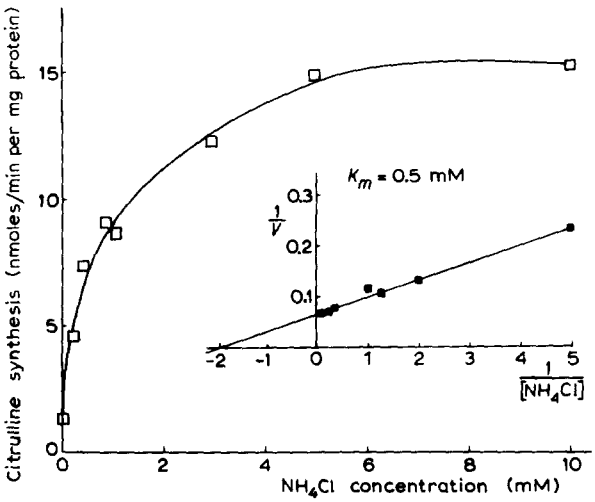

Fig. 2. Effect of NH_4Cl concentration on the rate of citrulline formation in mitochondria incubated in the presence of FCCP and oligomycin. Experimental conditions as in Fig. 1 with the exception that 5 mM ATP and various concentrations of NH_4Cl were added. Values shown are means of two experiments.

(i) glutamate oxidation proceeds *via* the deamination pathway, (ii) energy is produced *via* both substrate-level phosphorylation and oxidative phosphorylation. If the substrate-level phosphorylation is the only energy source, then phosphopyruvate carboxylase has a much higher capacity than the nucleoside diphosphate kinase for the intramitochondrial GTP, resulting in the inhibition of citrulline formation. This is probably due to the inhibitory effects of ADP on the conversion of GTP to ATP by nucleoside diphosphate kinase[32,33].

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