CONTROL OF THE RATE OF CITRULLINE SYNTHESIS BY SHORT-TERM CHANGES IN *N*-ACETYLGLUTAMATE LEVELS IN ISOLATED RAT-LIVER MITOCHONDRIA

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1. Introduction

Carbamoyl-phosphate synthase (ammonia) (EC 2.7.2.5) (CPS) has an absolute requirement for Mg^{2+} ions [1] and for the cofactor N-acetylglutamate (AGA) [2]. The possibility of regulation of CPS activity by variations in the concentration of mitochondrial-free Mg^{2+} ions was discussed [3]. Synthesis of AGA from acetylCoA and glutamate is a mitochondrial process [4–6]. According to [7] changes in mitochondrial AGA, for instance in response to changes in the protein content of the diet, occur only slowly because no breakdown of AGA in the mitochondria occurs and because the mitochondrial membrane seems to be impermeable to AGA [8].

In isolated rat-liver mitochondria citrulline production from added ornithine, ammonia and bicarbonate was preceded by a lag period of 5–10 min when glutamate was present as respiratory substrate [8] (cf. [3,9]). The rate of citrulline formation decreased by 50% when a combination of glutamate plus malate was used as the respiratory substrate instead of glutamate alone [10].

These observations led us to examine in more detail the relationship between the rate of citrulline production in isolated liver mitochondria, the activity of CPS and the concentration of mitochondrial AGA over relatively short time intervals. The results obtained suggest that the lag in citrulline production is due to activation of CPS by a fairly rapid production of AGA in the mitochondria. The inhibitory effect of malate on citrulline production is correlated with decreased CPS activity and a decreased AGA concentration. Moreover, it is shown that part of the AGA synthesized in the presence of glutamate is recovered in the extramitochondrial fluid.

2. Materials and methods

Liver mitochondria from rats fed on a standard diet were prepared as in [3].

Incubations were carried out at 25°C in closed 25 ml counting vials in a medium (1.2 ml) containing the following standard components: 75 mM Tris–HCl (pH 7.4), 15 mM KCl, 1 mM EGTA, 5 mM potassium phosphate, 16.6 mM $KHCO_3$, 10 mM ornithine, 10 mM NH_4Cl, 3 mM ATP and 25 mM mannitol. The gas phase was 95% O_2 + 5% CO_2.

The relative activity of CPS (see below) was measured at the end of the incubation as follows: 1 ml suspension was transferred to an Eppendorf centrifuge tube containing 0.1% (final concentration) Triton X-100, 5 μg oligomycin, 20 μmol $MgCl_2$, 10 μmol ATP and 5 μmol $NaH^{14}CO_3$ (spec. radioact. 0.1 μCi/μmol). The final volume was 1.2 ml and the incubation was continued in the closed tube at 25°C. The reaction was stopped by transferring 1 ml reaction mixture into a test-tube containing 0.3 ml 14% (w/v) $HClO_4$. Protein was removed by centrifugation. After removal of residual $^{14}CO_2$ by heating of the extract, the samples were counted by liquid scintillation counting in a solution containing per liter: 600 ml toluene, 200 ml alcohol, 200 ml Triton X-100, 25 mg 1,4-di-[2-(5-phenyloxazolyl)] benzene and 2 g 2,5-di-phenyloxazole. [^{14}C]Bicarbonate incorporation into citrulline was obtained by subtracting the values for incorporation in the absence of ornithine

from those in its presence. The incorporation of [^{14}C]bicarbonate into [^{14}C]citrulline in 14 min was used as a measure of CPS activity.

After lysis of the mitochondria [^{14}C]citrulline production was not linear but declined for 3–5 min until a low, constant rate was obtained. This effect may be due to release of AGA from CPS on dilution of the enzyme (cf. [11]). Thus the measurements of CPS activity can only be used for qualitative comparisons.

The following procedure was used to measure the AGA content of the mitochondrial suspension. At the end of the incubation, 1 ml suspension was transferred to each of a series of Eppendorf tubes containing 0.1% Triton X-100, 5 μg oligomycin and 0–50 nmol AGA. After 20 min at 25°C to allow a new equilibrium to be reached between CPS-bound and free AGA, synthesis of [^{14}C]citrulline was started by adding 5 μmol NaH$^{14}CO_3$, 20 μmol $MgCl_2$ and 10 μmol ATP. The final volume was 1.2 ml. [^{14}C]Citrulline production under these conditions was linear for at least 30 min (not shown). As a routine, the reaction was stopped after 20 min with $HClO_4$. Synthesis of [^{14}C]citrulline was a linear function of the amount of AGA added at least up to about 50 nmol. Endogenous AGA could then be estimated by extrapolation.

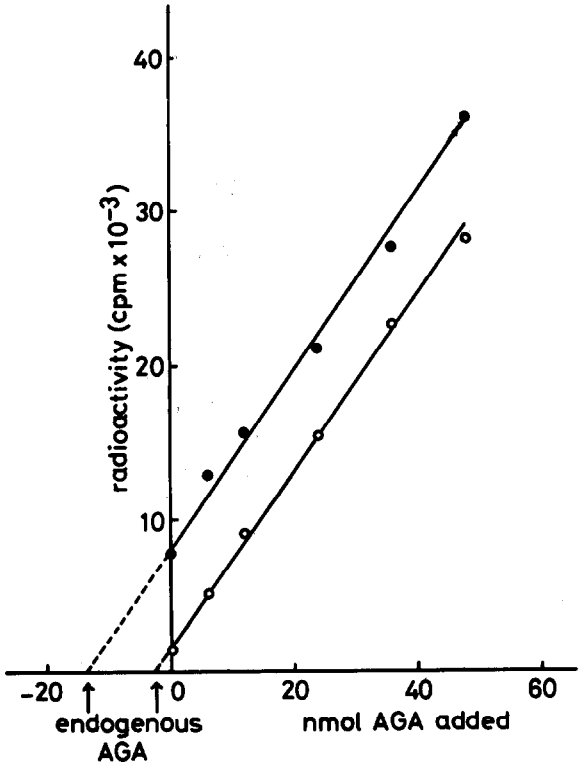

Fig.1. Stimulation of mitochondrial AGA production by glutamate. Mitochondria (3.8 mg protein/ml) were incubated for 12 min in a medium containing the standard components. AGA was determined as in section 2. (o——o) Control; (•——•) 10 mM glutamate present.

Figure 1 gives the results of a typical experiment showing the effect of glutamate on synthesis of AGA in intact rat-liver mitochondria. The intercepts on the abscissa represent the amount of endogenous AGA (2.6 nmol in the control, 13.6 nmol in the presence of glutamate).

Intramitochondrial AGA was measured by centrifuging 1 ml mitochondrial suspension at the end of the incubation in an Eppendorf microcentrifuge (Model 3200) for 20 s and dissolving the mitochondrial pellet in 1 ml medium containing the standard components (see above) plus 5 μg oligomycin, 0.1% Triton X-100 and 0–50 nmol AGA. The rest of the procedure was as described above.

Citrulline at the end of the initial incubation (prior to addition of Triton X-100) was measured as in [12].

Mitochondrial protein was determined by the biuret method [13].

3. Results

Figure 2 shows that after a lag period citrulline synthesis from added NH_3, ornithine and bicarbonate was high in the presence of glutamate as the ATP-generating substrate, but 50% lower when malate was present as well as glutamate. These results confirm the data in [8,10]. In addition, fig.2 demonstrates that malate had very little inhibitory effect when added to the mitochondria after they had been exposed to glutamate for 13 min.

Measurements of the relative activity of CPS are given in table 1. The activity of CPS was very low in untreated mitochondria (line 4) and increased about 3-fold after incubation of the mitochondria for 14 min in the absence of added glutamate (line 1). With glutamate present, CPS activity was further enhanced; this effect was completely abolished by the presence

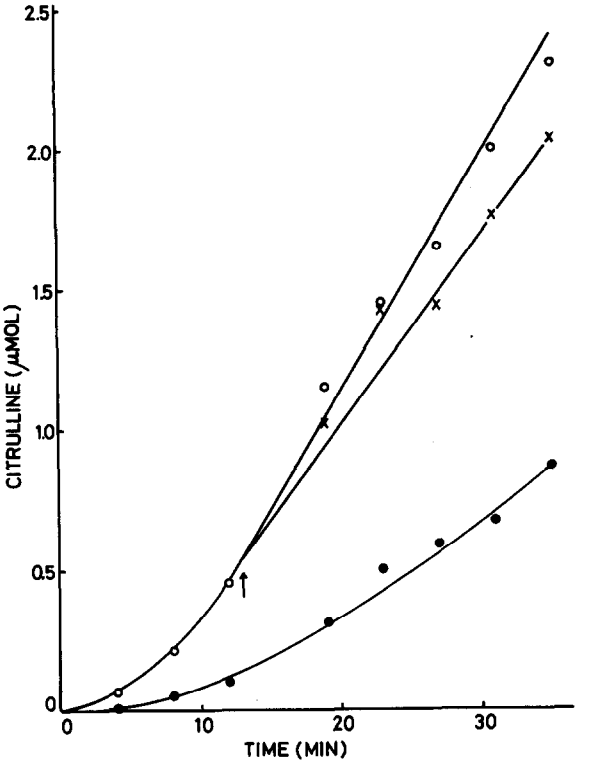

Fig.2. Effect of malate on citrulline synthesis in rat-liver mitochondria. Mitochondria (2.5 mg protein/ml) were incubated in a medium containing the standard components plus 10 mM glutamate and, where indicated, 10 mM malate. (o——o) Control; (•——•) malate present from the start; (X——X) malate added at 13 min.

of malate. Glutamate itself did not affect the activity measurement of CPS as shown by the fact that addition of glutamate together with Triton X-100 had no effect on CPS activity (table 1, line 5). The amount of citrulline produced by the mitochondria prior to the addition of Triton X-100 correlated well with the activity of CPS under the various conditions (table 1).

The experiments of table 2 show that the AGA content of the mitochondrial suspension was increased by glutamate and that this increase was abolished by malate. Furthermore, the amount of citrulline produced in the incubation period prior to the addition of Triton X-100 correlated with the AGA content of the suspension. When glutamate was replaced by β-hydroxybutyrate as the ATP-generating substrate both citrulline production (cf. [8]) and AGA content were low.

AGA synthesis in the presence of glutamate was linear for 20 min in one experiment (table 3, exp. 1). In other experiments, however, AGA formation declined earlier (table 3, exp. 2–4).

Table 3, experiments 2 and 4, demonstrates that almost all AGA in the suspension was intramitochondrial at zero time. However, after 12 min and 24 min a significant part of AGA was recovered in the extramitochondrial fluid.

Table 1
Citrulline synthesis and the activity of CPS in intact rat-liver mitochondria

Additions	ΔCitrulline (μmol)	Relative CPS activity (cpm)
None	0.37	5388
Glutamate	0.78	13 744
Glutamate + malate	0.36	3868
Triton X-100 at zero time	–	1658
Triton X-100 + glutamate at 14 min	–	4820

Mitochondria (3.2 mg protein) were incubated in a medium containing the standard components. Glutamate and malate, if added, were present at 10 mM. After 14 min, the mitochondria were lysed with 0.1% Triton X-100 and CPS activity after lysis was determined with [^{14}C]bicarbonate, as described in section 2

Table 2
Citrulline synthesis and the AGA content of intact rat-liver mitochondria

Exp.	Additions	(nmol/mg protein)	
		Δ Citrulline	AGA
1	None	180	0.7
	Glutamate	410	3.6
	Glutamate + malate	220	1.2
	β-Hydroxybutyrate	180	0.9
2	None	–	0.8
	Glutamate	–	3.2
	Glutamate + malate	–	1.1

Mitochondria (5.2 mg protein, exp. 1; 3.8 mg protein, exp. 2) were incubated for 12 min in a medium containing the standard components and, where indicated, 10 mM glutamate, 10 mM malate and 10 mM β-hydroxybutyrate. AGA was determined as described in the text (see fig.1)

Table 3
Time dependence of AGA synthesis and its distribution across the mitochondrial membrane

Exp.	Time (min)	nmol/mg protein	
		AGA_{total}	$AGA_{mitochondria}$
1	0	0.62	–
	5	1.3	–
	10	1.9	–
	20	3.3	–
2	0	0.62	0.56
	12	2.3	1.2
	24	2.5	1.1
3	0	–	–
	12	2.4	1.1
	24	3.2	1.3
4	0	0.91	0.91
	12	2.9	1.3
	24	2.9	1.3

Rat-liver mitochondria (2–4 mg protein/ml) were incubated in a medium containing the standard components. Total and mitochondrial AGA was measured according to the procedure described in section 2. In exp. 4, NH_3 and ornithine were omitted from the incubation medium and were later added together with [^{14}C]bicarbonate during the assay of AGA

4. Discussion

The rate of citrulline production in isolated mitochondria incubated with NH_3, ornithine, bicarbonate and succinate (+ rotenone) for a few minutes is directly correlated [7] with the amount of AGA present in the mitochondrial preparation at the start of the incubation, the latter being dependent on the protein content of the diet. It was concluded [7] that CPS activity was rate-limiting for citrulline under these conditions.

Our data provide direct evidence that during a relatively short period of incubation at 25°C, CPS activity can be altered by changes in the mitochondrial AGA concentration.

The inhibition of AGA synthesis by malate (fig.2) is probably due to removal of acetyl-CoA. Indeed, malate had little effect if added 13 min after glutamate (fig.2) since at this time AGA was already synthesized.

There is no doubt that the lag of 5–10 min in citrulline synthesis in intact rat-liver mitochondria incubated with glutamate, first observed in [8], is due to synthesis of AGA. The rate of AGA production found by us at 25°C is at least 0.1 nmol/min/mg mitochondrial protein. This value agrees well with

that of 0.25 nmol/min/mg protein, measured in rat-liver mitochondria at 37°C [7], but is much higher than the value of 4 nmol/30 min/20 mg protein, at 37°C [5] for isolated mouse-liver mitochondria. It must be stressed that our experiments were performed in the absence of arginine, which activates AGA synthetase [4–6], so that the true capacity for AGA production may be underestimated.

Synthesis of AGA occurs in the mitochondrial matrix [6]. The breakdown of AGA occurs in the cytosol [7,14,15]. Consequently efflux of AGA from the mitochondria is required. Because intramitochondrial CPS is inaccessible to extramitochondrial AGA [8] it was concluded [7] that changes in the intramitochondrial AGA concentration occur over relatively long time intervals, e.g., by induction or repression of AGA synthetase in response to changes in the diet. However, our results indicate that transport of AGA out of the mitochondria does take place. Thus, we suggest that, in vivo, regulation of CPS by variations in the mitochondrial AGA concentration occurs over relatively short time intervals.

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