EVIDENCE FOR THE EXISTENCE OF AN ORNITHINE/CITRULLINE ANTIPORTER IN RAT LIVER MITOCHONDRIA

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1. Introduction

In the urea cycle in rat liver, the enzymes carbamoyl phosphate synthase and ornithine transcarbamylase are situated in the mitochondria while the other enzymes of the cycle are cytoplasmic. Thus during the operation of the urea cycle, it is necessary for ornithine to enter the mitochondria and for citrulline to leave. These two amino acids are presumably transported by specific carrier systems, but the systems involved have not been adequately characterised.

Citrulline penetrates the mitochondrial membrane relatively slowly at low concentrations. Accordingly, it was originally suggested that citrulline may cross the mitochondrial membrane rapidly only via an ornithine/citrulline antiport system [1]. Gamble and Lehninger [2] presented evidence which they interpreted to show the existence of an electrogenic ornithine uniporter and a separate citrulline uniporter. However, Bryla and Harris [3] later showed that the transport of ornithine was not energy-dependent and was not linked in an obligatory manner to citrulline transport. McGivan et al. [4] showed that in the absence of citrulline, ornithine enters the mitochondrial matrix via an ornithine/H^+ antiport system.

It is possible that two ornithine transport systems exist: an ornithine/H^+ antiporter [4] and an ornithine/citrulline exchange system. In this paper, a method for loading mitochondria with citrulline is described, and evidence for an ornithine/citrulline antiporter is presented.

2. Experimental

Rat liver mitochondria were prepared by conventional methods in a medium containing 0.3 M mannitol, 5 mM Tris–HCl and 1 mM EGTA, pH 7.4. For many of the experiments described, mitochondria were loaded with ^{14}C-labelled citrulline by the following method. Mitochondria (100–150 mg protein) were incubated with stirring at pH 7.4 and 20°C for 15 min in a hypotonic medium containing (final concentrations) 60 mM mannitol, 20 mM Tris–HCl, 0.5 mM EGTA, 1 mM citrulline, 5 μCi [^{14}C-ureido]-L-citrulline and 50 μg rotenone in a total volume of 10 ml. The suspension was cooled and centrifuged at 10 000 × g for 10 min at 4°C. The supernatant was discarded, and the pellet resuspended in a small volume of cold isotonic preparation medium and used within 10 min.

For the measurement of citrulline efflux, aliquots of the citrulline-loaded mitochondria (2–3 mg protein) were added to 1 ml of preparation medium containing 5 μg rotenone together with the appropriate other additions at 4°C. The incubations were terminated by centrifugation at 10 000 × g on an Eppendorf Zentrifuge Model 3200. The pellet and supernatant were separated, deproteinised with perchloric acid (3.5% w/v final concentration) and assayed for radioactivity by liquid scintillation counting. The radioactivity in the pellet was corrected for counts in the extramitochondrial pellet water by determining the volume of extramitochondrial water carried down using ^{3}H-labelled sucrose as a marker in parallel experiments. This correction was usually <10% of the total pellet counts.

For the measurement of ornithine uptake described in table 2, a modification of this method was employed. Mitochondria were loaded with various concentrations of citrulline by incubation in the hypotonic medium described above except that the radioactive citrulline was omitted. The suspension

was then cooled, but not centrifuged. Aliquots of this suspension were incubated at 4°C in a medium containing (final concentrations) 50 mM mannitol, 50 mM KCl, 20 mM Tris–HCl, 2 mM K phosphate, 10 μg/ml rotenone, 5 μCi/ml [^{3}H]sucrose as a marker of the extramitochondrial water and 0.5 mM amino oxyacetate to inhibit ornithine aminotransferase. 1 mM L-ornithine-HCl was added together with 0.5 μCi/ml [U-^{14}C]L-ornithine HCl, and the mitochondria were separated after various times by centrifugation through silicone oil as described previously [4]. Intramitochondrial ornithine was taken as ornithine in the total pellet corrected for that in the extramitochondrial water. Radiochemicals were purchased from The Radiochemical Centre, Amersham, UK.

3. Results

3.1. *Loading of liver mitochondria with citrulline*

One approach to the identification of an antiport system in the mitochondrial membrane is to preload the mitochondria with one substrate of the postulated exchange and to observe the efflux of this compound induced by the addition of the second substrate. This approach has led to the characterisation of the glutamate/aspartate antiporter [5]. To study ornithine/citrulline exchange in liver mitochondria, it was necessary to devise a method for loading mitochondria with citrulline.

Citrulline when added at low concentrations enters mitochondria slowly [1]. Although it is possible to load mitochondria with citrulline by incubating them in isotonic citrulline [2], such mitochondria will not retain citrulline once the external citrulline is removed. It was found that if mitochondria were allowed to swell in a hypotonic medium, added citrulline penetrated the membrane rapidly at low concentrations. On centrifuging the mitochondria and resuspending in a citrulline-free isotonic medium the mitochondria retained citrulline for several minutes if incubated in the cold, although citrulline was lost rapidly at room temperature. It appeared therefore that, provided all experiments were performed in the cold, this system could be used to study citrulline efflux. Mitochondria loaded in this way in a medium containing 1 mM [^{14}C]citrulline contained 1.24 ± 0.11 nmol of [^{14}C]citrulline/mg protein (mean ± S.E.M. of 11 preparations) on resuspension, of which 0.9 nmol/mg came out of the mitochondria on addition of 5 mM unlabelled citrulline.

3.2. *Ornithine-induced efflux of citrulline from citrulline-loaded mitochondria*

Fig.1 shows the efflux of citrulline from mitochondria loaded with 1 mM [^{14}C]citrulline as described above. At 4°C the rate of citrulline efflux was relatively slow but this was increased by the addition of ornithine. It should be noted that under the conditions used, no conversion of ornithine to citrulline could occur since there was no possibility of ATP formation in the presence of rotenone. Also, since the [^{14}C]citrulline was labelled only in the ureido group, there could be no exchange of isotope between citrulline and ornithine. Fig.1 therefore represents a true efflux of citrulline on addition of ornithine and may be taken to indicate an antiport of these two amino acids across the membrane.

The specificity of this process is shown in table 1. [^{14}C]Citrulline efflux was promoted by the addition of 5 mM unlabelled citrulline, by ornithine and also

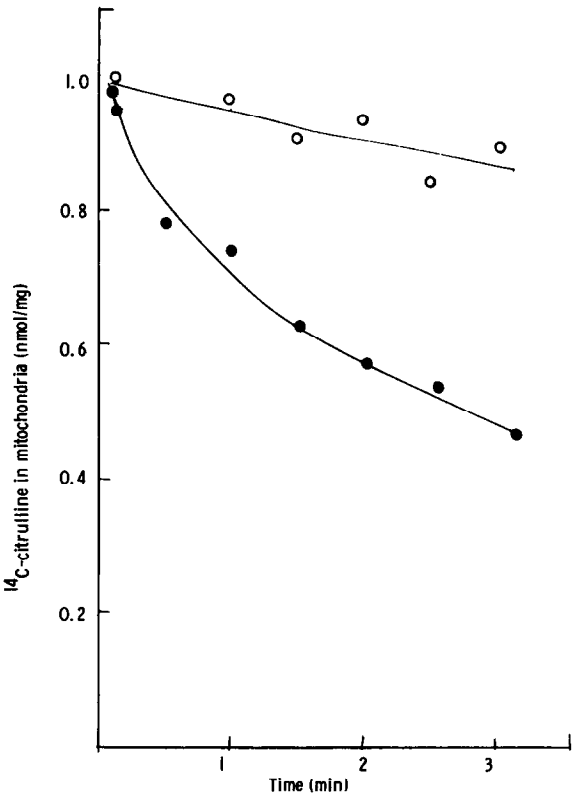

Fig.1. Time course of [^{14}C]citrulline efflux from mitochondria. Mitochondria were loaded with 1 mM [^{14}C]citrulline and incubated at 4°C as described in Section 2. –o–o–, no additions; –•–•–, + 5 mM ornithine-HCl.

Table 1
Effect of various compounds on citrulline efflux from citrulline-loaded mitochondria

Additions	[^{14}C]Citrulline released in excess of control (nmol/mg protein)
None	0
Ornithine (5 mM)	0.324 ± 0.03 (11)
Citrulline (5 mM)	0.57 ± 0.02 (4)
Lysine (5 mM)	0.61 ± 0.023 (4)
6-amino caproate (5 mM)	0.15 ± 0.08 (4)
5-amino valerate (5 mM)	<0.05
4-amino butyrate (5 mM)	<0.05
Histidine (5 mM)	<0.05

Mitochondria were preloaded with 1 mM [^{14}C]citrulline as described in Section 2. The loaded mitochondria were incubated with the appropriate additions for 90 s at 4°C. The values shown are the citrulline released in the presence of the appropriate addition minus that released in the same time with no additions. Results are the mean ± S.E.M. of the number of observations in brackets. The amount of citrulline released in the absence of additions varied between mitochondrial preparations in the range 0.02–0.08 nmol/mg in 90 s

by lysine. Lysine was more effective than ornithine. Various analogues of lysine and ornithine were tested; of these, only 6-amino caproate showed any effect on citrulline efflux. Citrulline efflux either in the presence or absence of ornithine was not stimulated by the addition of succinate to energise the mitochondria (results not shown). The rate of efflux of citrulline in response to the addition of ornithine was a function of ornithine concentration. It was found that the rate of citrulline efflux was half maximal at an external concentration of approx. 0.2 mM ornithine as judged from measurements of efflux after 90 s.

3.3. *Uptake of [^{14}C]ornithine into citrulline-loaded mitochondria*

It has been established that ornithine is able to enter mitochondria independently of the presence of citrulline via an ornithine/H^+ antiport system. If in addition to this system, there exists also an ornithine/citrulline exchange system, the uptake of ornithine into citrulline-loaded mitochondria should be higher than into control mitochondria. An experiment designed to test this possibility is shown in table 2. Mitochondria were loaded with various concentrations of unlabelled citrulline and 1 mM ^{14}C-labelled ornithine was added. It was found that the uptake of ornithine was maximal after 30 s. When the mitochondria were not preloaded with citrulline, a certain amount of ornithine was taken up, as shown previously [3,4]. In preloaded mitochondria, more ornithine was taken up, and the extra ornithine entry depended on the concentration of citrulline in the preloading medium. In a control experiment, mitochondria were not preloaded, but were preincubated with citrulline for 1 min before the addition of ornithine. Under these conditions the uptake of ornithine was unaffected, indicating that the extra ornithine uptake depended on the internal rather than external citrulline concentration.

4. Discussion

In this report, evidence is presented for the existence of an antiport mechanism in liver mitochondria which catalyses the influx of ornithine and the efflux of citrulline. The existence of such a system is deduced both from measurements of citrulline efflux from citrulline-loaded mitochondria and also from separate measurements of ornithine uptake into such mitochondria. The ornithine/citrulline antiporter appears also to catalyse citrulline/citrulline and lysine/citrulline exchange. Competition between ornithine and lysine for this carrier may in part account for the fact that lysine inhibits urea synthesis in isolated hepatocytes [6].

Ornithine is positively charged at neutral pH while citrulline has no net charge. An ornithine/citrulline

Table 2
Uptake of [^{14}C]ornithine into citrulline-loaded and control mitochondria

(1) Citrulline-loaded mitochondria [citrulline] in loading medium [mM]	[^{14}C]Ornithine in pellet (nmol/mg)
0	2.90 ± 0.22 (10)
0.5	3.72 ± 0.17 (6)*
1.0	4.95 ± 0.15 (7)**
5.0	7.16 ± 0.23 (7)**
(2) Control mitochondria [Citrulline] added to mitochondria (mM)	
0	2.42 ± 0.34 (3)
0.5	2.31 ± 0.05 (3)
1.0	3.04 ± 0.46 (3)
5.0	2.63 ± 0.22 (3)

The experiment was carried out as described in Section 2. In Exp. 1, the mitochondria were preloaded with citrulline and subsequently incubated with 1 mM [^{14}C]ornithine for 1 min. The results are the mean ± S.E.M. of the number of observations in brackets. In Exp. 2, mitochondria were not preloaded, but were incubated with citrulline for 1 min before the addition of ornithine and centrifuged after a further period of 1 min. *$P < 0.05$; **$P < 0.001$ vs. control

antiporter could therefore catalyse either the net influx of positive charge, or an electroneutral exchange if one H^+ were transported in the same direction as the citrulline molecule. The system used in the present experiments did not allow investigation of this point. The fact that succinate did not stimulate ornithine/citrulline exchange may indicate that this exchange is not electrogenic.

Investigation of ornithine transport by measurements of [^{14}C]ornithine uptake may lead to misleading results. Thus, if amino oxyacetate is not present, some transamination of [^{14}C]ornithine with endogenous oxoglutarate may occur, and the intramitochondrial radioactivity will not reflect intramitochondrial ornithine exclusively [4]. Use of [^{14}C]-ornithine at very low concentrations, especially in a medium of low ionic strength, requires a method for discrimination between ornithine transported and that bound to the membrane [4]. Further, although the ornithine cation appears to cross the energised mitochondrial membrane electrogenically at very high ornithine concentrations [2], this is not the case at low ornithine concentrations [3,4]. Similarly, citrulline penetrates the membrane rapidly at high concentrations [2] but not at low concentrations. In certain earlier studies on ornithine and citrulline transport, these factors were not sufficiently appreciated.

It appears that ornithine crosses the mitochondrial membrane by two distinct transport systems. One system is an ornithine/citrulline antiporter and this is required for the function of the urea cycle. The other is an electroneutral ornithine/H^+ exchange which is independent of citrulline movement. The latter system is required for the communication of the matrix enzyme ornithine-oxoglutarate aminotransferase with cytosolic ornithine. A combination of these two carrier systems is compatible with the present data and also with much of the data of Bryla and Harris [3]. These workers found that citrulline added to mitochondria increased ornithine uptake, in contrast to the present report. However, it is possible that some of the added citrulline may have entered the mitochondria under their conditions.

Interpretation of the finding of these workers that arginine inhibits ornithine uptake requires the demonstration of the absence of arginase from the mitochondrial preparation. In our hands, mitochondria isolated by conventional methods contain considerable activity of arginase as a contaminant. Generation of unlabelled ornithine by the arginase reaction would be expected to reduce the uptake of ^{14}C-labelled ornithine into mitochondria.

References

[1] Chappell, J. B., McGivan, J. D. and Crompton, M. (1972) in: The Molecular Basis of Biological Transport (Woessner, J. F. jr and Huijing, F. eds) pp. 55–81, Academic Press, London.
[2] Gamble, J. G. and Lehninger, A. L. (1973) J. Biol. Chem. 248, 610–618.
[3] Bryła, J. and Harris, E. J. (1976) FEBS Lett. 72, 331–336.
[4] McGivan, J. D., Bradford, N. M. and Beavis, A. D. (1977) Biochem. J. 162, 147–156.
[5] LaNoue, K. F. and Tischler, M. E. (1974) J. Biol. Chem. 249, 7522–7528.
[6] Krebs, H. A., Lund, P. and Stubbs, M. (1976) in: Gluconeogenesis (Hanson, R. W. and Mehlman, M. A. eds) pp. 269–292.