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KINETICS OF GLUTAMINE EFFLUX FROM LIVER MITOCHONDRIA LOADED WITH THE ^{14}C-LABELED SUBSTRATE

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Glutamine transport across the inner membrane of rat liver mitochondria was studied by the method of loading the organelles with [^{14}C]glutamine and by measuring efflux of the metabolite at 0°C. The release of [^{14}C]glutamine from loaded mitochondria was prevented by mersalyl, whereas the efflux was started by the addition of glutathione. The rate of glutamine efflux from the mitochondria was measured by the inhibitor stop technique with mersalyl plus *N*-ethylmaleimide. It was found that up to 10 mM glutamine there is no significant activity of glutaminase, whereas at about 20 mM of the substrate the enzyme is activated. The rate of the efflux measured after the addition of the optimal amount of glutathione was 10 nmol glutamine/min per mg protein. This is 5-times faster than the rate of glutaminase activity at 0°C. The pH optimum of glutamine carrier is between 6.5 and 7.0. Low concentration of succinate inhibits the efflux due to formation of pH gradient in coupled mitochondria, whereas a higher concentration of succinate inhibits the carrier directly. 2-Oxoglutarate and glutamate strongly inhibit the rate of glutamine efflux, the inhibition by glutamate being very pronounced at its physiological concentration. D-Glutamine does not inhibit the rate of the efflux, indicating that the transport of L-glutamine is stereospecific.

Introduction

Since glutaminase (EC 3.5.1.2) is an intramitochondrial enzyme [1], the study of regulation of glutamine deamidation is associated with the problem of the transport of the substrate across the inner mitochondrial membrane. The question of whether glutamine transport is the rate-limiting step for the activity of the enzyme or not was put forward especially in connection with the problem of regulation of renal ammoniagenesis in metabolic acidosis, but a satisfactory explanation is still lacking [2]. Glutamine transport in liver mitochondria has attracted much less attention since liver glutaminase has been far less investigated than the kidney enzyme. However, recently much effort has been made towards understanding the function and regulation of the liver glutaminase [3]. It has been shown [4] that mersalyl, which is an SH-blocking agent, inhibits glutamine influx into rat liver mitochondria, suggesting the presence of a carrier-mediated transporting system, except in that we could not find any other experimental data related to this problem. The investigation of the transport in kidney mitochondria, although very intensive in the last few years [5–7], has not provided satisfactory results, mainly because under all reported experimental conditions glutaminase activity was quite high so that metabolism of glutamine interfered with transport process. Besides, measurement of the influx of a metabolite is usually connected with technical difficulties due to a large surface-to-volume ratio of mitochondria and volume change of the organelles. For this reason an accurate determination of substrate concentration in the matrix space is quite

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difficult, especially if it does not accumulate. Taking all this into account we assumed that loading of the mitochondria with [^{14}C]glutamine and measurement of its efflux would be a more convenient method for the study of kinetics of glutamine transport. Also, in order to keep glutaminase activity as low as possible, all experiments were carried out at 0°C and without inorganic phosphate which is an activator of the enzyme. The results obtained with rat liver mitochondria and preliminary experiments with rat kidney mitochondria suggest that this method represents a new and promising approach to the study of glutamine transport in mitochondria. Part of this communication was presented at the Second International Workshop on Ammoniagenesis in Athens.

Materials and Methods

Rat liver mitochondria were isolated as described elsewhere [8] except that the last washing was performed with a KCl medium comprising 100 mM KCl/10 mM Tris-HCl (pH 7.0)/0.5 μg/ml rotenone. L-[U-^{14}C]Glutamine (Amersham International, U.K.) and [U-^{14}C]glutamic acid (the product of glutamine hydrolysis) were separated by column chromatography on Dowex 1-X8, and their radioactivity was measured by a liquid scintillation counter. Mitochondrial protein was determined by a biuret method [9].

Loading of the mitochondria with [^{14}C]glutamine. 1.0 ml of a thick suspension of the mitochondria (approx. 50 mg protein) was mixed with 0.3 ml 40 mM [U-^{14}C]glutamine (0.4 Ci/mol) and incubated 5 min at 0°C. This was followed by the addition of 0.25 ml 20 mM mersalyl (100 nmol/mg protein) and 0.1 ml 0.5 M $MgCl_2$. The mitochondria were washed twice with the sucrose medium which was used for their isolation [8] but without EGTA, and once with the KCl medium. The final volume was adjusted to 1.5 ml and used immediately for the transport experiments. Without addition of mersalyl all ^{14}C label was lost during washing procedure. The amount of [^{14}C]glutamine in the mitochondrial pellet was 20–30-times higher than in the same volume of the supernatant.

Release of [^{14}C]glutamine from loaded mitochondria. 50 μl of mitochondrial suspension (approx. 2 mg protein) were added to 1 ml of the KCl medium at 0°C and the efflux of [^{14}C]glutamine was started by the addition of glutathione. It was found that glutathione completely removes the inhibition of glutamine uptake caused by mersalyl and that the mitochondria thereafter show functional integrity [10]. The mitochondria were separated from the medium by centrifugation in an Eppendorf centrifuge and radioactivity in the supernatant fraction was measured.

The measurement of the rate of [^{14}C]glutamine efflux from the mitochondria by the inhibitor stop technique. The efflux of [^{14}C]glutamine started by the addition of glutathione was rapidly inhibited by the addition of mersalyl plus *N*-ethylmaleimide. This was applied for the measurement of the rate of glutamine release from the mitochondria.

Results and Discussion

The amount of glutathione required for the release of [^{14}C]glutamine from the mitochondria

It was expected that on the molar basis less amount of GSH would be required in order to remove the inhibition by mersalyl. However, the measurement of the percentage of the release of [^{14}C]glutamine as the function of the amount of GSH revealed that this was not the case. Fig. 1 shows that much higher amount of GSH was required compared to the amount of mersalyl added to the mitochondria in order to release glutamine completely. This suggest that SH-groups of the carrier which are blocked by mersalyl are not easily accessible to glutathione. Incubation of the mitochondria in a hypotonic KCl medium (60 mM KCl) did not have significant influence on the release of glutamine (not shown). Further experiments showed that the release is also time-dependent.

Capacity of the mitochondria to take up glutamine

The mitochondria were loaded with increasing concentration of [^{14}C]glutamine (from 4.7 to 75.6 mM), and radioactivity was measured in the mitochondrial extract after separation from [^{14}C]glutamic acid. Fig. 2 shows that rat liver mitochondria have a large capacity for taking up glutamine. However, at a concentration of about 20 mM of glutamine there is a pronounced activa-

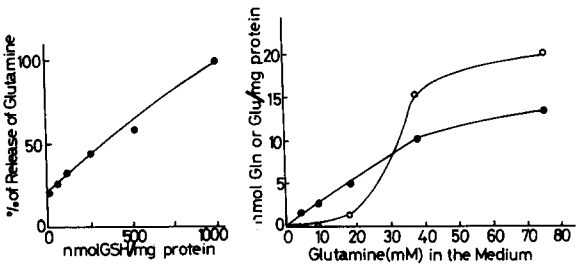

Fig. 1. (Left.) Release of [^{14}C]glutamine from rat liver mitochondria by the addition of different amounts of GSH. The time of incubation was 5 min.

Fig. 2. (Right.) The amount of [^{14}C]glutamine and [^{14}C]glutamic acid in rat liver mitochondria loaded with different concentrations of [^{14}C]glutamine. ● [^{14}C]glutamine; ○, [^{14}C]glutamic acid.

tion of glutaminase by the substrate, even though the temperature is 0°C and the medium is without phosphate. It should be noted that the glutamine curve is hyperbolic, whereas the glutamate curve is sigmoidal. Hydrolysis of glutamine at higher concentrations creates great difficulty in the determination of the K_m of the carrier for the substrate. This is especially so if we know that the carrier is inhibited by glutamate (Fig. 7). Preliminary experiments indicated that the carrier is not saturated up to 10 mM glutamine.

Measurement of the rate of glutamine efflux from the mitochondria

The fastest rate of glutamine efflux from the mitochondria was obtained by the addition of 3 μmol GSH/mg protein (Fig. 3). At 5 mM intramitochondrial glutamine the rate of efflux was 10 nmol glutamine/min per mg protein. Similar values were obtained by measurement of glutamine influx into the mitochondria. This, together with the finding that the influx and the efflux are equally sensitive to the inhibition by mersalyl, suggests that we are dealing with the same carrier molecule which facilitates equilibration of glutamine across the inner mitochondrial membrane in both directions. It should be pointed out that a greater amount of inhibitors is required (6 μmol mersalyl plus 6 μmol of *N*-ethylmaleimide per mg protein) compared to the amount of GSH in order to achieve very rapid inhibition of the efflux of glutamine. *N*-Ethylmaleimide was added because, in contrast to mersalyl, it penetrates the mitochondrial membrane easily and reacts with intramitochondrial GSH.

pH dependence of glutamine efflux and effects of succinate and an uncoupler

Determination of the pH dependence of the rate of glutamine efflux from the mitochondria showed that the pH optimum of the carrier lies between 6.5 and 7.0 (Fig. 4). The addition of an uncoupler does not change the pH profile except in the alkaline part of the pH curve. However, the addition of succinate leads to a significant change in the pH profile of the carrier so that a flat pH curve is obtained. This is probably due to formation of pH gradient in the presence of succinate (more alkaline inside) which results in the decrease of activity of the carrier, especially in more acidic range of the pH scale. For this reason the addition of FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) abolished the effect of succinate at lower concentrations (Fig. 5). Removal of the inhibition by FCCP was less efficient at higher concentrations of succinate due probably to direct inhibition of the carrier by the anion.

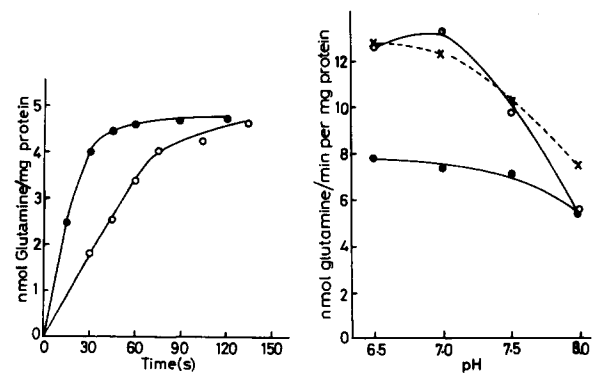

Fig. 3. (Left.) The rate of the efflux of [^{14}C]glutamine from liver mitochondria after addition of two different amounts of GSH. ○, 1.5 μmol GSH/mg protein; ●, 3.0 μmol GSH/mg protein.

Fig. 4. (Right.) Influence of different pH of the medium on the rate of [^{14}C]glutamine efflux from liver mitochondria and effect of FCCP and succinate on the pH profile of the carrier. ○, control; ×, plus 1 μM FCCP; ●, plus 1 mM succinate.

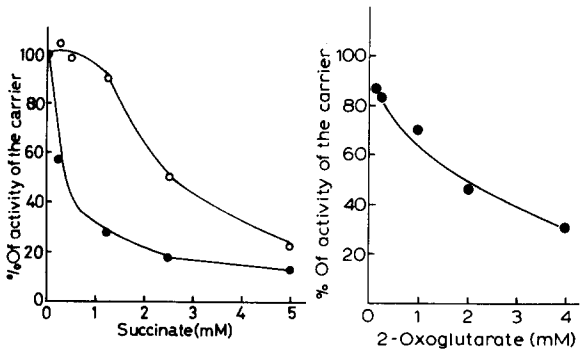

Fig. 5. (Left.) Effect of succinate in coupled and uncoupled liver mitochondria on the rate of [^{14}C]glutamine efflux. ●, succinate; ○, succinate plus 1 μM FCCP.

Fig. 6. (Right.) Inhibition of the rate of [^{14}C]glutamine efflux from liver mitochondria by 2-oxoglutarate.

The effect of 2-oxoglutarate, glutamate and D-*glutamine on the rate of glutamine efflux*

It was shown by Goldstein and collaborators [11] that glutamine carrier of rat kidney mitochondria is inhibited by a physiological concentration of 2-oxoglutarate, indicating that the change of the level of this metabolite can modulate the rate of glutamine transport and deamidation. In accordance with this finding, we obtained inhibition of the liver mitochondrial carrier by 2-oxoglutarate, but the effect was not so pronounced at low concentrations of the metabolite (Fig. 6). However, the addition of glutamate, even at physiological concentration (approx. 2.5 mM) inhibited the rate of glutamine efflux 60% (Fig. 7). This suggests that glutamate may be physiological regulator of glutamine transport in rat liver mitochondria. Experiments with D-glutamine showed that the carrier is not sensitive to this isomeric form of glutamine, indicating that the transporting protein is stereospecific (Fig. 7).

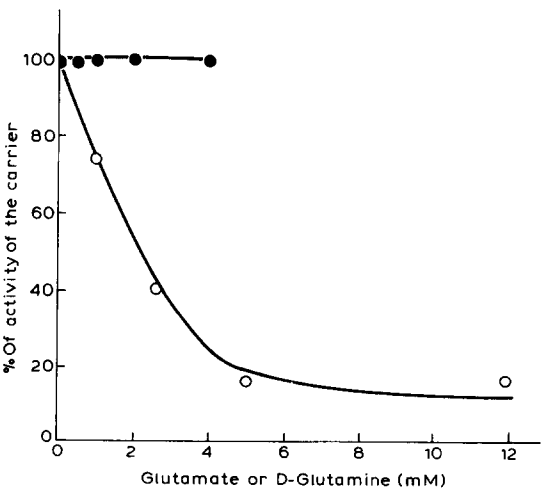

Fig. 7. Effect of glutamate and D-glutamine on the rate of [^{14}C]glutamine efflux from liver mitochondria. ○, glutamate; ●, D-glutamine.

TABLE I

ACTIVITY OF GLUTAMINE CARRIER AND GLUTAMINASE IN RAT LIVER AND RAT KIDNEY MITOCHONDRIA

Experimental conditions for the measurement of activity of the carrier in both kind of mitochondrion were as described in Materials and Methods. Glutaminase was assayed in the same KCl medium but in the presence of the optimal concentration of phosphate and at the optimal pH for enzyme activities.

	nmol glutamine or glutamate per min per mg protein	
	0°C	30°C
Liver glutamine carrier	10.00	—
Liver glutaminase	1.95	11.00
Kidney glutamine carrier	7.20	—
Kidney glutaminase	0.65	15.00

Comparison of the activity of glutamine carrier and glutaminase in rat liver and rat renal mitochondria

Measurement of activity of the carrier and glutaminase showed that the rate of the transport is 5- to 10- times faster than the rate of glutamine hydrolysis by glutaminase. Activity of glutaminase was measured in the presence of the optimal concentration of P_i and at optimal pH of the medium (pH 7.4 in the case of liver, and pH 8.0 in the case of kidney enzyme). This suggests that activity of the carrier is not the rate-limiting step in the deamidation of glutamine in both kinds of mitochondrion. However, in the light of the finding that the carrier is inhibited by several physiological intermediates, especially by glutamate, this conclusion may by premature and should await further experimental support.

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