Preferential glutamine uptake in rat brain synaptic mitochondria

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Glutamine uptake has been studied in purified rat brain mitochondria of synaptic or non-synaptic origin. It was taken up by an active saturable transport mechanism, with an affinity two-times higher in synaptic than in non-synaptic mitochondria (K_m = 0.45 and 0.94 mM, respectively). V_{max} of uptake was 7-times higher in synaptic mitochondria (V_{max} = 9.2 and 1.3 nmol/min per mg protein, respectively). Glutamine transport was found to be inhibited by L-glutamate (IC_{50} = 0.64 mM) as well as thiol reagents (mersalyl, *N*-ethylmaleimide). It is suggested that differential uptake of glutamine in mitochondria of synaptic or non-synaptic origin may be a major mechanism in the regulation of the synthesis of the neurotransmitter glutamate.

Glutamine uptake *Purified mitochondria* *Glutamate*

1. INTRODUCTION

Glutamine appears to be the preferential precursor of the two neurotransmitters γ-aminobutyric acid (GABA) in GABAergic neurons and glutamate in glutamatergic neurons [1]. The metabolic pathways of glutamate and glutamine in the CNS are closely interrelated and compartimentalized: glutamate is mainly confined to neurons, while glutamine is synthesized in glial cells from endogenously formed glutamate or after reuptake of exogenous glutamate [2–4]. Results concerning glutamine uptake in both glial and neuronal cells with characterization of the commonly called glutamine-glutamate-GABA cycle are abundant [1,3–6]. In contrast, there are few data trying to evaluate the transport of glutamine in brain mitochondria [7]. Hydrolysis of glutamine into glutamate by phosphate activated glutaminase is mainly localized in mitochondria [2]. Thus glutamate formation can be regulated either by the activity of that enzyme or by the entry of glutamine into mitochondria. Neuronal glutaminase activity which may be related to the synthesis of the neurotransmitter glutamate has been shown to be higher than in glial cells. The present study is the first report of glutamine transport in purified synaptic mitochondria compared to non-synaptic ones.

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2. MATERIALS AND METHODS

2.1. *Preparation of mitochondria*

Mitochondrial fractions were isolated according to the method of Lai et al. [8] modified by Rendon and Masmoudi [9]. All steps in this procedure were carried out at 4°C. Adult rats of Wistar strain were used in all experiments. Eight rats were killed by decapitation in each preparation. The forebrains of the animals were finely minced and rapidly washed in isolation buffer (0.32 M sucrose, 1 mM EDTA K^+, 10 mM Tris-HCl, pH 7.4) to remove contamination by blood. A 20% (w/v) homogenate in isolation buffer was made in a motor-driven Potter homogenizer with 3 up-down strokes. The homogenate was then diluted to 10%

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(w/v) with isolation buffer and centrifuged at 1100 × *g* for 5 min. The supernatant was centrifuged at 17000 × *g* for 10 min to yield the crude mitochondrial pellet containing synaptosomes (P2 fraction). P2 was washed by resuspending in isolation buffer and centrifuged at 17000 × *g* for 20 min. The pellet was resuspended in 30 ml of isolation buffer by manually homogenizing. 9 ml of this suspension was layered onto a dicontinuous Ficoll gradient (7.5, 13%) and centrifuged at 100000 × *g* for 30 min in a Beckman type 28 SW rotor. The 7.5 or 13% Ficoll media contained 7.5 or 13% (w/v) Ficoll in isolation buffer. The myelin fraction was collected at the interface between the isolation buffer and 7.5% Ficoll medium, the synaptosomal fraction in the interphase between 7.5% and 13% Ficoll medium and the non-synaptic mitochondria at the bottom of the tube. The non-synaptic mitochondria were washed in 20 ml of isolation buffer and resuspended manually in 3 ml of the same medium. The synaptosomal fraction was diluted with 3 vols of isolation buffer centrifuged at 18500 × *g* for 10 min. The pellet was homogenized manually and lysed with 40 ml of 6 mM Tris-HCl (pH 8.1) for 90 min under constant agitation. The lysate was centrifuged at 75000 × *g* for 15 min. The pellet was resuspended in 3 ml of isolation buffer and 1 ml of this suspension layered over a discontinuous iso-osmotic gradient of Percoll (10, 20, 40 amd 60%). Centrifugation was carried out at 37000 × *g* during 20 min in a Beckman type 40 angle headed rotor.

The synaptic mitochondria banded in the interphase between 20 and 40% Percoll. The non-synaptic mitochondria were layered in the same type of gradient and were recovered at the same level as the synaptic ones. The 2 fractions were sucked out with Pasteur pipettes, diluted with 20 vols of isolation buffer and spun at 12500 × *g* for 10 min. Fractions were separated from coated silica particles by repeated washing with 20 ml of the isolation buffer. Finally pellets were resuspended in isolation buffer to give a concentration of 5–10 mg of protein per ml. The fractions were characterized by enzyme markers as described in [9].

2.2. *Glutamine transport studies*

For glutamine uptake measurement, L-[3,4-^{3}H]glutamine (0.2 μCi/tube; 0.1–2.5 mM final concentration) was added to 200 μl of a medium containing 115 mM KCl, 3.6 mM Hepes (pH 7.4), 0.48 mM $MgSO_4$, 10 mM succinate and 1 μg/ml rotenone. Incubation was started at 4°C by adding to the medium 20 μl of mitochondria suspension. At the end of the incubation, mitochondria were rapidly separated by centrifugation (5000 × *g* during 2 min) through a mixture of bromodecane and bromododecane oil (1:1, v/v) into 10% perchloric acid. After neutralization of perchloric acid with NaOH, radioactivity in the pellet was measured by scintillation spectrometry (Intertechnique Spectrometer SL 40). Assays were run in parallel with [^{14}C]sucrose in order to determine the volume of medium bound to the mitochondria pellet.

The effects of thiol reagents (mersalyl and *N*-ethylmaleimide [NEM]) and of related compound (L-glutamate, α-oxoglutarate) on glutamine uptake were tested with Ficoll purified mitochondria at 4°C. After 5 min pre-incubation of the mitochondria with one of these agents, transport was initiated by adding radioactive glutamine (0.2 μCi/tube; 2 mM final concentration). The reaction was stopped 3 min later by centrifugation.

In other experiments, the metabolism of accumulated labelled glutamine was measured. Ficoll purified mitochondria were incubated during 1–4 min in a medium containing 0.25 mM L-[^{3}H]glutamine (0.4 μCi/tube). After centrifugation the pellet was dissolved in a small volume of 80% ethanol. Electrophoretic separation of the supernatant was performed according to the method of Sarhan et al. [10] using a Camag type 67520 electrophoresis apparatus. The radioactive spots were then scraped off, eluted in a small volume of water and counted in a scintillation spectrometer.

Protein content of mitochondrial suspension was determined by the method of Lowry et al. [11] using bovine plasma albumin as standard.

3. RESULTS

3.1. *Time course and temperature dependence of glutamine transport*

Uptake of 0.25 mM L-[3,4-^{3}H]glutamine was studied in Ficoll purified mitochondria as shown in fig.1. Uptake was found to be linear at both 4°C and 23°C until 4 min incubation. The rate of up-

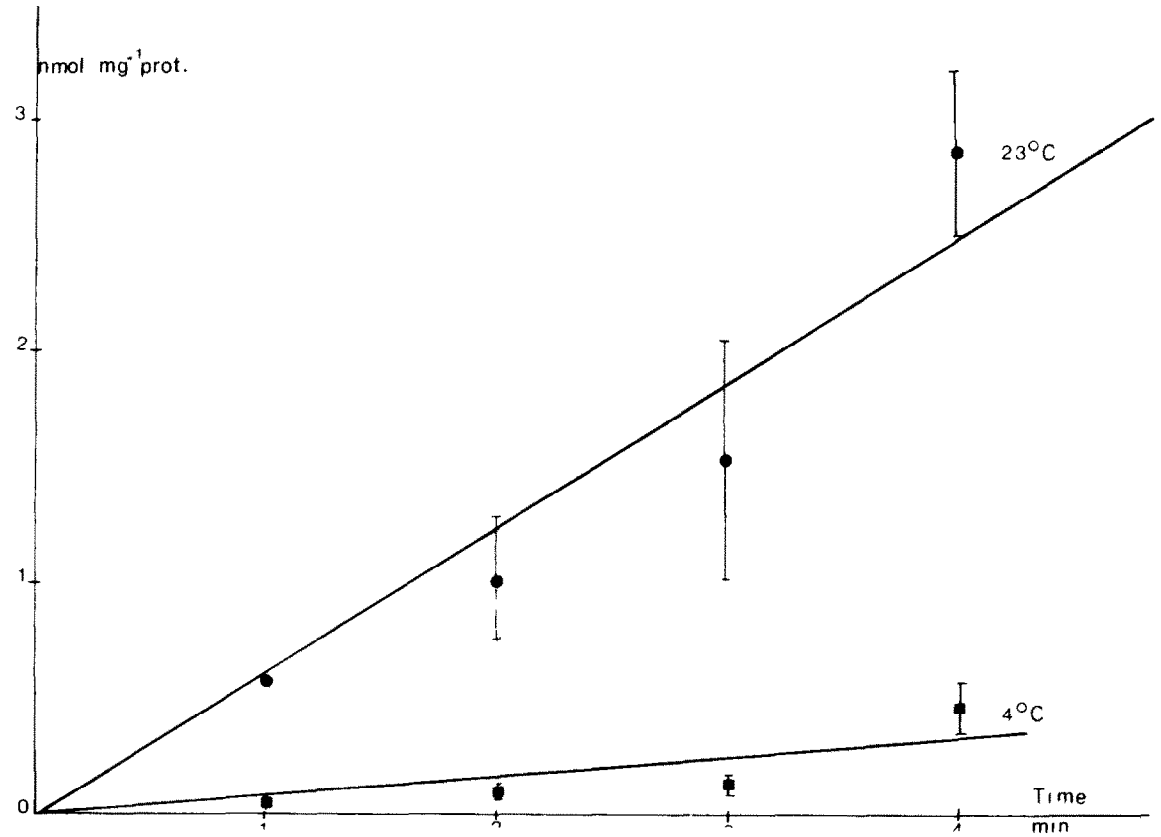

Fig.1. Time course of 0.25 mM glutamine uptake by Ficoll purified mitochondria incubated at 4°C and 23°C in a medium containing 115 mM KCl, 10 mM succinate, 3.6 mM Hepes (pH 7.4), 0.48 mM $MgSO_4$ and 1 μg/ml rotenone.

take was higher at 23°C than at 4°C. Results are expressed in nmol · mg protein^{-1} after subtraction of the blank values.

3.2. *Kinetic characteristics of glutamine transport*

Experiments were carried out on synaptic and non-synaptic mitochondria. The concentration of unlabelled glutamine added to the medium ranged between 0.1 and 2.5 mM. The reaction was stopped after 3 min of incubation. The rate of uptake is expressed in nmol · min^{-1} · mg protein^{-1} after subtraction of the blank at each concentration. In both types of mitochondria the uptake was found to occur by an active transport system that reached a plateau at 1 mM concentration (fig.2 and 3). The K_m value for non-synaptic mitochondria was 2-times higher than that for synaptic mitochondria. The most striking difference between both types of mitochondria was that V_{max} of uptake was found to be 7-times higher in synaptic mitochondria than in non-synaptic ones (table 1).

3.3. *Effect of thiol reagents and related compounds on the uptake of glutamine*

Both thiol reagents (mersalyl and NEM, 0.1 mM and 16 μM, respectively) were potent inhibitors of glutamine uptake. Moreover α-oxoglutarate (2.5 mM) and glutamate (2.5 to 20 mM) inhibited uptake by 55 and 68%, respectively (table 2). IC_{50} value for glutamate inhibition was found to be 0.64 mM (fig.4).

3.4. *Distribution of the radioactivity after [^{3}H]-glutamine uptake*

The time course of the specific activity of glutamate and glutamine was measured after [^{3}H]glutamine uptake (not shown). The results showed that 80–90% of the radioactivity was recovered as glutamine between 1 and 4 min incubation.

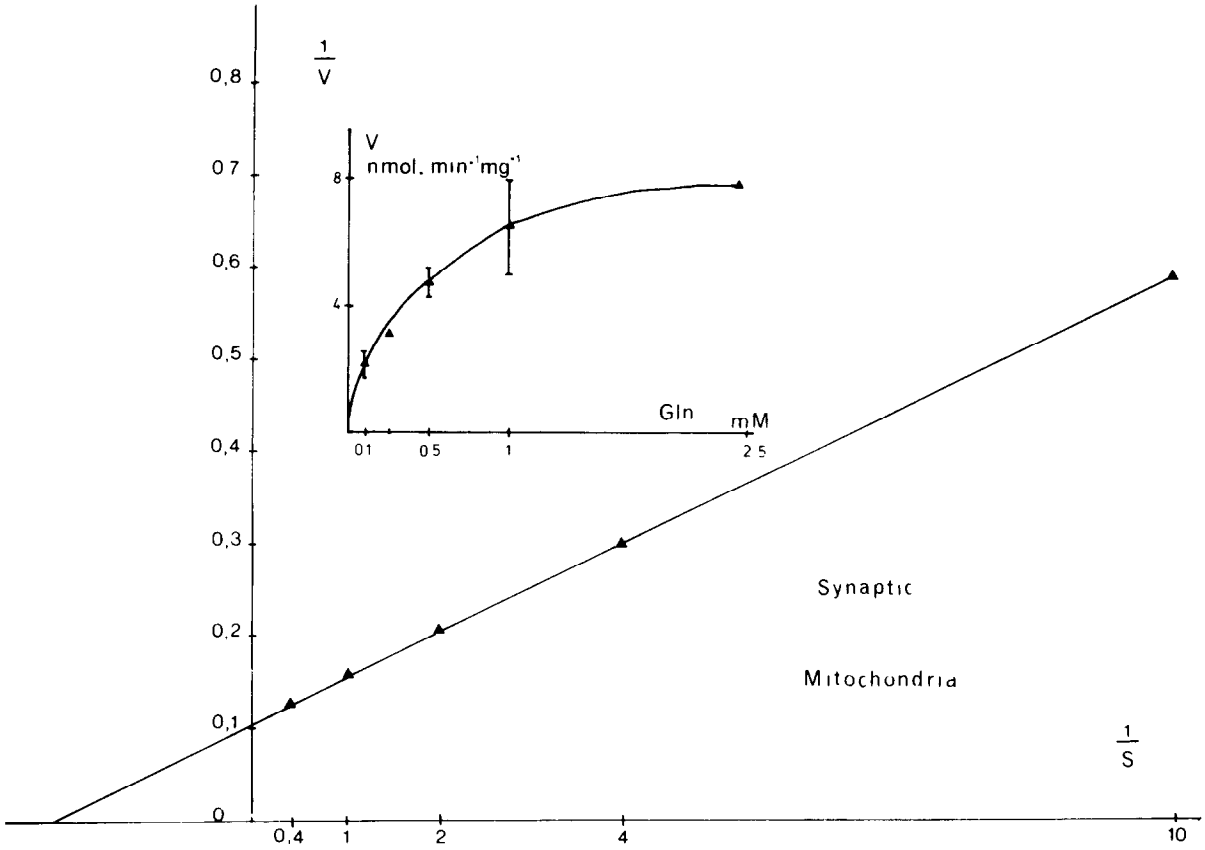

Fig.2. Kinetics of the initial uptake of L-[3,4-^{3}H]glutamine by synaptic mitochondria shown as a Lineweaver-Burk plot. The points are the mean of 3–7 determinations ± SE for 3 min uptake at 4°C. Inset: the rate of uptake (V) in nmol · min^{-1} · mg protein^{-1} is plotted against glutamine concentration (S) in mM.

Table 1

Kinetic constants of the uptake of L-[3,4-^{3}H]glutamine

	K_m (μM)	V_{max} (nmol · min^{-1} · mg protein^{-1})
Non-synaptic mitochondria	941 ± 239 (18)	1.326 ± 0.173
Synaptic mitochondria	447 ± 209 (26)	9.211 ± 1.674

The values of K_m and V_{max} and their standard deviations were computed with a PDP8 using Cleland program [17]. The number of experimental values is shown between parentheses

Table 2

Effect of thiol reagents and compounds related to glutamine on the uptake of L-[3,4-^{3}H]glutamine in Ficoll purified mitochondria

	% inhibition
Mersalyl, 0.1 mM	74 ± 1.4
Mersalyl, 0.4 mM	79 ± 6
NEM, 16 μM	86 ± 8
NEM, 32 μM	82 ± 3
α-Oxoglutarate, 2.5 mM	68 ± 2.2
Glutamate, 2.5 mM	55 ± 5
Glutamate, 5 mM	57 ± 11
Glutamate, 10 mM	61 ± 4.5
Glutamate, 20 mM	58 ± 11

The results are expressed as percentage inhibition ± SD of three experiments. [^{3}H]Glutamine uptake was measured during 3 min at 4°C following a 5 min preincubation with each tested compound

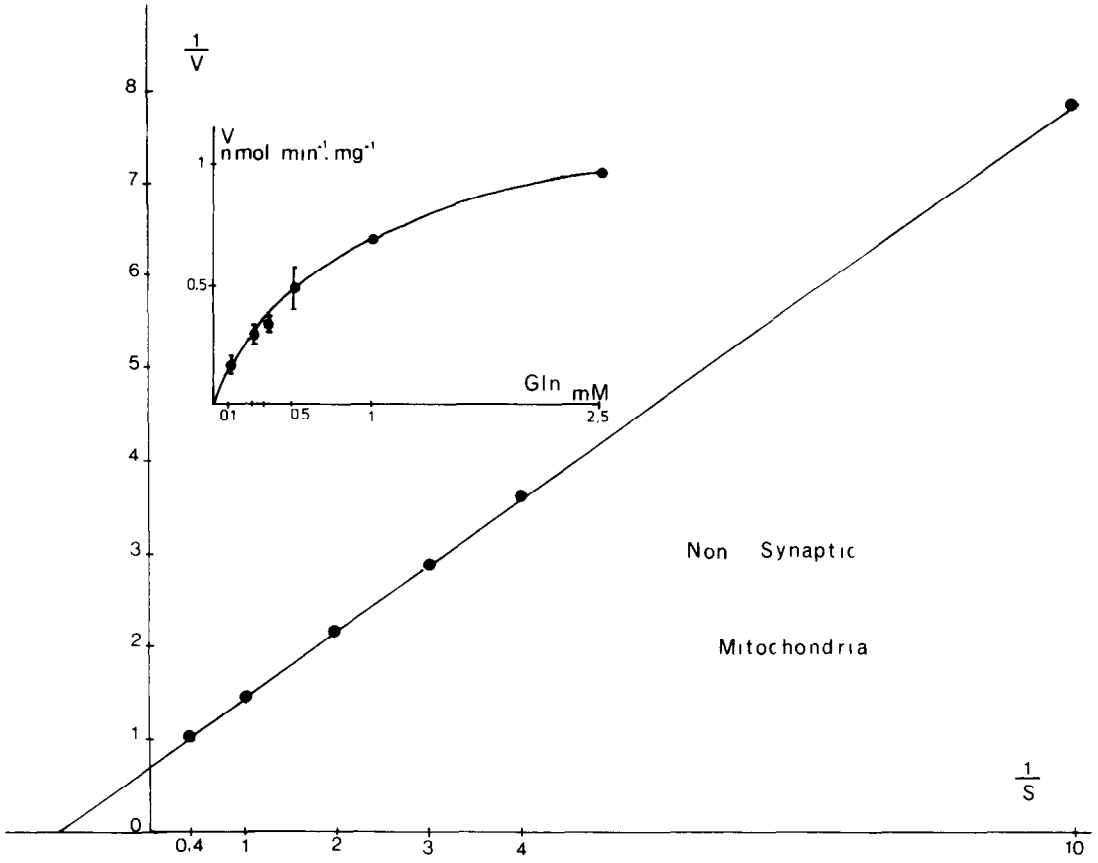

Fig.3. Kinetics of the initial uptake of L-[3,4-^{3}H]glutamine by non-synaptic mitochondria shown as a Lineweaver-Burk plot. The points are the mean of 3–7 determinations ± SE for 3 min uptake at 4°C. Inset: the rate of uptake (V) in nmol · min^{-1} · mg protein^{-1} is plotted against glutamine concentration (S) in mM.

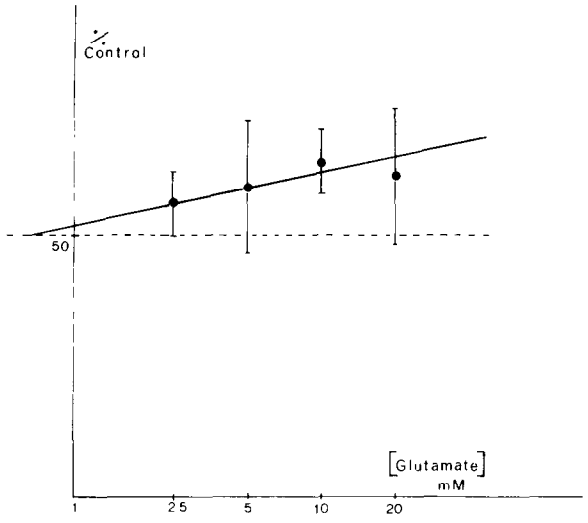

Fig.4. Determination of IC_{50} value for glutamate inhibition of glutamine uptake in Ficoll purified mitochondria. Each point is the mean of 3 experimental data in which the glutamate concentration varied between 2.5 and 20 mM. The inhibitor was added during 5 min preincubation and [^{3}H]glutamine uptake was measured after a 3 min incubation. [^{3}H]Glutamine uptake as percent of the control is plotted on a probability scale against inhibitor concentrations (log scale) to obtain an IC_{50} value.

4. DISCUSSION

Because of the intramitochondrial location of glutaminase and of other enzymes related to glutamate metabolism, knowledge of the characteristics of glutamine transport in mitochondria, may help to understand the synthesis of the neurotransmitter glutamate. The present report suggests that the transport of glutamine into mitochondria of nerve terminals may be related to the regulation of glutamate formation.

Our results indicate that glutamine transport into brain mitochondria is not the result of a simple diffusion process. The experimental data are consistent with a carrier mediated mechanism, as has been reported by Minn [7] in non-synaptic mitochondria. Glutamine uptake into purified synaptic mitochondria compared to non-synaptic ones was never described. According to our results, synaptic mitochondria showed a strikingly higher uptake capacity: the affinity was 2-times higher and the velocity 7-times higher than in the non-synaptic ones. This finding suggests that the two species of mitochondria could have distinct properties for glutamine uptake. Synaptic mitochondria (directly involved in neurotransmission), may have a larger distribution of specific glutamine carrier with a higher affinity, when compared to perikaryal and glial mitochondria. The characterization by Kvamme et al. [12,13] of two species of mitochondrial phosphate activated glutaminase having distinct regulatory properties are in accordance with this hypothesis. Furthermore, Dagani et al. [14] have reported significant differences in specific activities of enzymes measured in non-synaptic and synaptic mitochondria.

Various reports have suggested a glutamine entry into mitochondria correlated to a glutamate efflux. As described in the present study, the inhibitory effect of mersalyl on glutamine transport suggests that the integrity of some membrane hydrophobic thiol groups is needed. Moreover, NEM which was reported to inhibit glutamate transport in liver and brain mitochondria [15], also produced a strong inhibitory effect on glutamine uptake. Glutamate and α-oxoglutarate were both potent inhibitors of glutamine uptake. These results are in accordance with an antiporter process, that exchanges glutamate for glutamine. A similar scheme was already proposed for glutamine transport in kidney mitochondria [16]. However, in a previous study [7], NEM was reported to be without effect on glutamine transport by brain mitochondria, suggesting two independent carrier mechanisms for glutamine and glutamate transport. Further evaluation, using the addition of a specific inhibitor of glutaminase, changes of pH in the medium as well as characterization of glutamine metabolism in separate synaptic and non-synaptic mitochondria, is needed to confirm this hypothesis.

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