

Metabolism of [U-¹³C]Glutamine and [U-¹³C]Glutamate in Isolated Rat Brain Mitochondria Suggests Functional Phosphate-Activated Glutaminase Activity in Matrix

Lasse K. Bak · Elżbieta Ziemińska ·
Helle S. Waagepetersen · Arne Schousboe ·
Jan Albrecht

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Abstract One of the forms of phosphate activated glutaminase (PAG) is associated with the inner mitochondrial membrane. It has been debated whether glutamate formed from glutamine in the reaction catalyzed by PAG has direct access to mitochondrial or cytosolic metabolism. In this study, metabolism of [U-¹³C]glutamine (3 mM) or [U-¹³C]glutamate (10 mM) was investigated in isolated rat brain mitochondria. The presence of a functional tricarboxylic (TCA) cycle in the mitochondria was tested using [U-¹³C]succinate as substrate and extensive labeling in aspartate was seen. Accumulation of glutamine into the mitochondrial matrix was inhibited by histidine (15 mM). Extracts of mitochondria were analyzed for labeling in glutamine, glutamate and aspartate using liquid chromatography-mass spectrometry. Formation of [U-¹³C]glutamate from exogenous [U-¹³C]glutamine was decreased about 50% ($P < 0.001$) in the presence of histidine. In addition, the ¹³C-labeled skeleton of [U-¹³C]

glutamine was metabolized more vividly in the tricarboxylic acid (TCA) cycle than that from [U-¹³C]glutamate, even though glutamate was labeled to a higher extent in the latter condition. Collectively the results show that transport of glutamine into the mitochondrial matrix may be a prerequisite for deamidation by PAG.

Keywords Mass spectrometry · Glutaminase · PAG · Heterogeneous

Abbreviations

LC-MS Liquid chromatography-mass spectrometry
PAG Phosphate-activated glutaminase
TCA Tricarboxylic acid

Introduction

Glutamine provided by astrocytes is generally accepted to be the main precursor for both neurotransmitter glutamate and GABA biosynthesis. The enzyme responsible for deamidation of glutamine to glutamate, phosphate-activated glutaminase (PAG) is a mitochondrial enzyme existing in two forms, namely an inner membrane-bound and a soluble form exhibiting differential kinetic profiles and sensitivity to inhibitors and activators; the membrane-bound form seems to be the active form of the enzyme [1, 2]. PAG exhibits a higher activity in synaptic mitochondria as compared to non-synaptic and astrocytic mitochondria [3–5].

It has been debated whether glutamate formed in the glutaminase reaction has direct access to mitochondrial or cytosolic metabolism. In case of glutamate biosynthesis, inhibitors of aspartate aminotransferase as well as the dicarboxylate carrier both being entities of the malate-aspartate shuttle [6] partly prevent exocytotic release [7].

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Lasse K. Bak and Elżbieta Ziemińska contributed equally to the experimental work described in this paper.

L. K. Bak (✉) · H. S. Waagepetersen · A. Schousboe
Department of Pharmacology and Pharmacotherapy, Faculty
of Pharmaceutical Sciences, University of Copenhagen, 2
Universitetsparken, 2100 Copenhagen, Denmark
e-mail: laba@farma.ku.dk
URL: www.neurometabolism.dk

E. Ziemińska
Department of Neurochemistry, Medical Research Centre,
Polish Academy of Sciences, Warsaw, Poland

J. Albrecht
Department of Neurotoxicology, Medical Research Centre,
Polish Academy of Sciences, Warsaw, Poland

Thus, it was concluded that glutamate synthesized from glutamine most likely gains access to the mitochondrial matrix. With regard to GABA it was shown using [^{13}C]glutamine as precursor that approximately 60% of GABA synthesis occurs via participation of the tricarboxylic acid (TCA) cycle [8]. In contrast, based on metabolic studies of ^{14}C -labeled glutamine in isolated brain mitochondria, it has been suggested that the catalytic activity of PAG is facing the inter-membrane space, i.e. glutamate is accessible in the cytosol [9]. This may be incompatible with results from a recent study employing isolated brain mitochondria and histidine to inhibit glutamine transport, showing that PAG activity may be functionally associated with the inner mitochondrial membrane facing the matrix side [10].

The present study further elaborates on this hypothesis by investigating the metabolism of exogenously applied [^{13}C]glutamine (3 mM), [^{13}C]glutamate (10 mM) or [^{13}C]succinate (2.5 mM) plus glutamate (10 mM) employing histidine (15 mM) to inhibit transport of glutamine into the mitochondrial matrix [3].

Experimental procedure

Materials

Female Wistar rats weighing 150–200 g were used in this study. The uniformly ^{13}C -labeled glutamate, glutamine and succinate were from Cambridge Isotope Laboratories (Cambridge, MA, U.S.A.). All other chemicals used were commercially available products of highest possible purity.

Isolation of mitochondria

A slightly modified method based on Lai and Clark [11] and Lai et al. [12] was employed for the isolation of rat cerebral mitochondria. Gray matter of cerebral cortex and hippocampus of 4–6 rats were homogenized in a Dounce-type glass homogenizer in ice-cold homogenization medium containing 225 mM mannitol, 75 mM sucrose, 0.5 mM EGTA, 10 mM Tris-HCl (pH 7.4). All steps were carried out at 4°C. The homogenate was diluted to 50 ml in homogenization medium (containing 1 mg/ml bovine serum albumin) and centrifuged at 2,000g for 4 min. The resulting supernatant was centrifuged at 12,500g for 10 min. The pellet was re-suspended in separation medium consisting of 0.12 M mannitol, 0.03 M sucrose, 25 μM EDTA and 3% ficoll and placed on top of double-concentrated separation medium in the centrifuge tubes. This preparation was centrifuged at 11,500g for 30 min. The pellet was re-suspended in homogenization medium and centrifuged at

12,500g for 10 min. This final pellet was re-suspended in a small volume of homogenization medium and used for the experiments. To evaluate the quality of the mitochondrial preparation, the respiratory control ratio was determined before each experiment and only preparations exhibiting values higher than 3.0 were employed for experiments. The typical values obtained were between 3.0 and 4.5.

Incubation of mitochondria

Isolated rat brain mitochondria (corresponding to 1.6–1.9 mg of protein per sample) were incubated at 25°C in a total volume of 1 ml in medium containing 100 mM KCl, 75 mM mannitol, 25 mM sucrose, 10 mM Tris-phosphate/10 mM Tris-HCl (pH 7.4), 50 μM EDTA, albumin (1 mg/ml), 1.25 mM ADP, and 0.5 mM pyruvate (as oxidative substrate). Mitochondria were incubated for 5 min in medium containing 3 mM [^{13}C]glutamine, 10 mM [^{13}C]glutamate or 2.5 mM [^{13}C]succinate plus 10 mM glutamate. Some experiments were performed in the presence of histidine (15 mM) to inhibit glutamine transport into the matrix [3]. The incubations were terminated by placing the samples on ice followed by centrifugation at 15,000g for 10 min. The pellet, representing mitochondria was extracted in 1 ml 70% v/v ethanol and centrifuged for 20 min at 20,000g. The resulting pellet was discarded and the supernatant was subsequently lyophilized.

Biochemical analysis

The lyophilized samples were reconstituted in water. The Phenomenex EZ:faast amino acid kit for LC-MS was used for analysis of labeling in glutamate and aspartate. LC-MS analyses were performed using a Shimadzu LCMS-2010 mass spectrometer coupled to a Shimadzu 10A VP HPLC system. Protein content of the individual suspensions of mitochondria were determined according to Lowry et al. [13] using the Bio-Rad DCProtein assay.

Data analysis

Data were analyzed employing Microsoft Excel 2003 and GraphPad Prism v4.01 software. All labeling data were corrected for natural abundance of ^{13}C by subtracting a sample of the relevant metabolite. Isotopic enrichment was calculated according to Biemann [14]. Data are presented as means \pm SEM and statistical differences are calculated by one-way ANOVA followed by Bonferroni post hoc test. A *P*-value of <0.05 was considered statistically significant.

To obtain a measure of total incorporation of ^{13}C label into aspartate, average percent of labeled carbon atoms was calculated, as initially introduced by Bak et al. [15]. Aspartate may contain anywhere between one and four ^{13}C atoms. To provide a measure of the total labeling of the aspartate pool, the percent of the individual isotopomers are first multiplied by the number of carbons labeled. Subsequently, these numbers are summed up and expressed as a percent of the total number of carbon atoms, denoted molecular carbon label (MCL, %). See the Results section for a description of labeling in aspartate derived from metabolism of the labeled precursors employed.

Results

The pattern and extent of ^{13}C -labeling in glutamate and aspartate can be employed as an indicator of TCA cycle metabolism, since in any given microenvironment glutamate and aspartate can be considered to be in equilibrium with α -ketoglutarate and oxaloacetate, respectively, due to the high activity of aspartate aminotransferase [16, 17]. The validity of this approach for intact systems has recently been challenged because of slow equilibration between the large cytosolic and the mitochondrial pool of glutamate, which is especially important for interpreting in vivo ^{13}C magnetic resonance spectroscopy studies [18]. However, this caveat does not apply for the present work employing

isolated mitochondria. Thus, when uniformly ^{13}C -labeled glutamine is deamidated to uniformly labeled glutamate, this label will enter the TCA cycle via conversion of glutamate to α -ketoglutarate (Fig. 1). α -Ketoglutarate is metabolized in the TCA cycle to uniformly labeled oxaloacetate which gives rise to quadruple labeled aspartate via transamination. Alternatively, oxaloacetate may condense with unlabeled acetyl-CoA forming quadruple labeled citrate and subsequently, triple labeled glutamate and double labeled aspartate may be formed. In the second turn of the TCA cycle glutamate will be double or mono labeled and aspartate mono labeled. Subsequent cycling in the TCA cycle will only give rise to mono labeled α -ketoglutarate and oxaloacetate and in turn glutamate and aspartate. Metabolism of $[\text{U-}^{13}\text{C}]$ succinate gives rise to similar isotopomers. Note that triple labeled aspartate (not shown in Fig. 1) may only be formed if labeled malate leaves the TCA cycle and re-enters as labeled acetyl-CoA in condensation with labeled oxaloacetate, a process known as pyruvate recycling [19].

TCA cycle metabolism was estimated in the presence of exogenous glutamate (10 mM), by employing $[\text{U-}^{13}\text{C}]$ succinate (2.5 mM). As expected, the detected glutamate labeling was low due to the presence of unlabeled glutamate, whereas aspartate to a large extent was uniformly labeled (Table 1). In contrast, the mono, double and triple labeling of aspartate was limited which may be due to the short (5 min) incubation time.

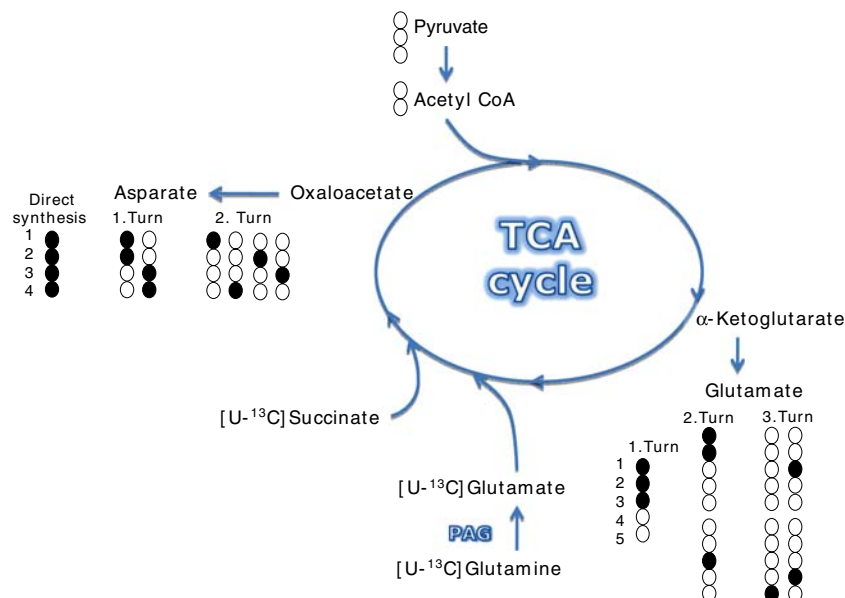


Fig. 1 Simplified scheme of TCA cycle metabolism of $[\text{U-}^{13}\text{C}]$ glutamine, $[\text{U-}^{13}\text{C}]$ glutamate or $[\text{U-}^{13}\text{C}]$ succinate and acetyl-CoA derived from pyruvate. The possible combinations of label in glutamate and aspartate during three turns of the TCA cycle are shown. In the first turn of the TCA cycle in which unlabeled acetyl-CoA condenses with uniformly labeled oxaloacetate, glutamate and

aspartate will be triple and double labeled, respectively. Subsequent turns will give rise to both unlabeled, mono and double labeled glutamate and mono labeled aspartate. Labeled carbon atoms are represented by black circles. PAG, phosphate-activated glutaminase; TCA, tricarboxylic acid

However, the presence of isotopomers other than uniformly labeled aspartate unequivocally demonstrates TCA cycle activity.

Employing [U-¹³C]glutamine (3 mM) as the substrate about 80% of the mitochondrial glutamine was uniformly labeled; regardless of the presence of 15 mM histidine (Table 2). Formation of uniformly labeled aspartate was most pronounced with only minor mono, double and triple labeling. Likewise, glutamate was primarily uniformly labelled. Conversion of [U-¹³C]glutamine to [U-¹³C]glutamate as well as MCL (%) of aspartate decreased by ~50% in the presence of the glutamine transport inhibitor histidine (Table 2 and Fig. 2).

Using [U-¹³C]glutamate (10 mM) as substrate, approximately 60% of the mitochondrial glutamate was uniformly labeled at the end of the incubation period (Table 3). Aspartate synthesized from labeled glutamate exhibited an MCL value which was lower than that of aspartate derived from labeled glutamine (Fig. 2).

Discussion

It has been suggested that PAG, which is localized in the inner mitochondrial membrane [4], is functionally associated with the outer face of this membrane, i.e. glutamate formed by deamidation is preferentially released to the

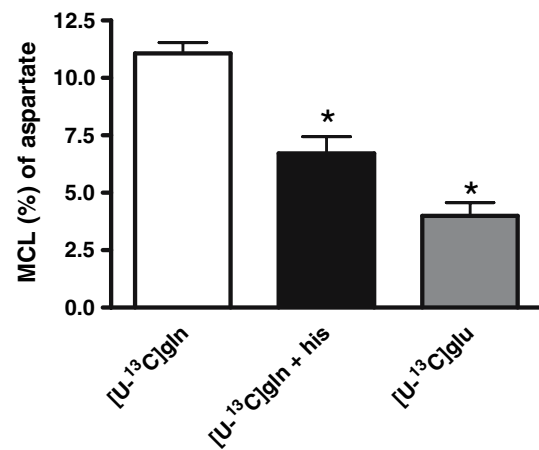


Fig. 2 Molecular carbon labeling (MCL; %) of mitochondrial aspartate. Rat brain mitochondria prepared as described in Experimental Procedures were incubated in the presence of [U-¹³C]glutamine (3 mM; open bar), [U-¹³C]glutamine and histidine (15 mM; black bar) or [U-¹³C]glutamate (gray bar). Label of aspartate in extracts of mitochondria was determined by LC-MS and MCL (%) was calculated (see Experimental Procedures). The values are averages of 5–8 determinations ± SEM. Statistically significant differences are determined by one-way ANOVA followed by Bonferroni post hoc test. *: significantly different from incubation in the presence of [U-¹³C]glutamine ($P < 0.05$)

inter-membrane space and is likely to leave the mitochondria [1]. However, this was challenged by the finding that histidine, an inhibitor of mitochondrial glutamine

Table 1 Labeling (%) of glutamate and aspartate in isolated rat brain mitochondria after incubation in the presence of 2.5 mM [U-¹³C]succinate plus 10 mM glutamate

Amino acid	Mono label	Double label	Triple label	Quadruple label	Quintuple label
Glutamate	0.5 ± 0.1	0.3 ± 0.1	0.7 ± 0.2	0.1 ± 0.0	0.7 ± 0.1
Aspartate	2.1 ± 0.1	1.1 ± 0.3	1.8 ± 0.2	32.2 ± 2.8	–

Rat brain mitochondria prepared as described in Experimental Procedures were incubated in the combined presence of [U-¹³C]succinate (2.5 mM) and glutamate (10 mM). Label of glutamate and aspartate in extracts of mitochondria was determined by LC-MS and expressed as percent mono, double, triple, quadruple or quintuple label (see Results section). The values are averages of 8 experiments ± SEM

Table 2 Labeling (%) of glutamate, glutamine and aspartate in isolated rat brain mitochondria after incubation in the presence of 3 mM [U-¹³C]glutamine or [U-¹³C]glutamine and 15 mM histidine

Amino acid	Condition	Mono label	Double label	Triple label	Quadruple label	Quintuple label
Glutamate	Control	0.6 ± 0.2	0.4 ± 0.2	0.5 ± 0.2	n.d.	15.0 ± 1.8
	15 mM His	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	n.d.	8.7 ± 0.4*
Glutamine	Control	n.d.	n.d.	n.d.	n.d.	84.4 ± 1.6
	15 mM His	n.d.	n.d.	n.d.	n.d.	80.8 ± 2.2
Aspartate	Control	2.7 ± 0.2	1.6 ± 0.3	0.9 ± 0.2	8.9 ± 1.6	–
	15 mM His	2.6 ± 0.1	1.1 ± 0.1	0.6 ± 0.03	5.8 ± 0.3	–

Rat brain mitochondria prepared as described in Experimental Procedures were incubated in the presence of [U-¹³C]glutamine (3 mM) or [U-¹³C]glutamine and histidine (15 mM). Label of glutamate, glutamine and aspartate in extracts of mitochondria was determined by LC-MS and expressed as percent mono, double, triple, quadruple or quintuple label (see Results section). The values are averages of 5–8 experiments ± SEM. Statistically significant differences are indicated by asterisks and determined by one-way ANOVA followed by Bonferroni post hoc test. * $P < 0.05$. n.d., not detectable

Table 3 Labeling (%) of glutamate and aspartate in isolated rat brain mitochondria after incubation in the presence of 10 mM [^{13}C]glutamate

Amino acid	Condition	Mono label	Double label	Triple label	Quadruple label	Quintuple label
Glutamate	Control	n.d.	n.d.	0.2 ± 0.03	n.d.	57.9 ± 8.4
Aspartate	Control	2.0 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	3.1 ± 0.5	–

Rat brain mitochondria prepared as described in Experimental Procedures were incubated in the presence of [^{13}C]glutamate (10 mM). Label of glutamate and aspartate in extracts of mitochondria was determined by LC-MS and expressed as percent mono, double, triple, quadruple or quintuple label (see Results section). The values are averages of seven experiments ± SEM. n.d., not detectable

transport [3], significantly decreased the content of glutamate, but not glutamine, in rat brain mitochondria incubated in the presence of glutamine [10]. Hence, it was concluded that the enzymatic activity of PAG was compromised by inhibition of glutamine transport into the mitochondrial matrix, as histidine does not inhibit PAG activity per se [3]. This notion is incompatible with the above mentioned association of PAG with the outer face of the inner mitochondrial membrane. It should be noted in this context that it was shown previously that the mitochondrial aspartate aminotransferase plays a functional role in biosynthesis of neurotransmitter glutamate [7], a mechanism requiring that glutamate produced in the PAG catalyzed hydrolysis gains access to the mitochondrial matrix. Interestingly, recent mathematical modelling of the scheme proposed by Palaiologos et al. [7] has provided additional evidence for its validity [20]. In addition, TCA cycle metabolism has been shown to play a significant role for biosynthesis of neurotransmitter glutamate using [^{13}C]glutamine [21].

The finding in the present study that histidine inhibited formation of uniformly labeled glutamate from exogenously supplied [^{13}C]glutamine, suggests that access to deamidation was reduced by inhibiting accumulation of glutamine into the mitochondrial matrix. Histidine by itself does not inhibit accumulation of glutamate [22, 23]. This observation supports the conclusion by Zieminska et al. [10] that PAG activity is functionally present in the mitochondrial matrix. The observation that histidine only inhibited approximately 50% of the conversion of glutamine to glutamate is in agreement with results obtained by Zieminska et al. [10] and Albrecht et al. [3] as histidine at the concentration employed (5 times excess) does not block glutamine transport completely. Formation of ^{13}C -labeled aspartate from [^{13}C]succinate indicated that the TCA cycle was active in these mitochondria. MCL (%) of aspartate derived from labeled glutamine was higher than that derived from labeled glutamate, even though the concentration of glutamate as substrate was 3 times that of glutamine. It should be noted, that about 60% of mitochondrial glutamate was uniformly labeled after incubation in the presence of [^{13}C]glutamate. This is in agreement with the accumulation of exogenous glutamate observed by Zieminska et al. [10] under similar conditions. These

observations indicate that glutamate formed via deamidation gains preferential access over exogenous glutamate to TCA cycle metabolism. In line with this, the decreased MCL (%) value of aspartate in the presence of histidine reflects decreased TCA cycle metabolism of ^{13}C -labeled glutamate derived from deamidation of labeled glutamine. It should be noted that MCL (%) is a relative measure and does not provide information on the actual amount of labeled aspartate formed. It is conceivable that the amount of mitochondrial glutamate and in turn aspartate decreased in the presence of histidine, as observed with regard to glutamate by Zieminska et al. [10]. However, a decrease in MCL (%) of aspartate does provide strong support for the notion that synthesis of labeled aspartate was decreased in the presence of histidine caused by reduced formation of labeled glutamate from glutamine catalyzed by PAG.

In conclusion, the previous suggestion that PAG is associated with the outer surface of the inner mitochondrial membrane [1] may need to be re-evaluated. The present study provides evidence that glutamine transport is a prerequisite for glutamate formation and further metabolism in the TCA cycle, altogether demonstrating that the active site of PAG must be facing the matrix side. Such functional localization may allow channeling of glutamate produced from glutamine directly into different metabolic pathways associated with e.g. energy metabolism or neurotransmitter replenishment. This could not be achieved in a well controlled manner if glutamate was synthesized in the inter-membrane space facing the outer membrane, thus being accessible in the cytosol as concluded by Roberg et al. [9].

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