Control and function of the transamination pathways of glutamine oxidation in tumour cells

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Parallel investigations of the transamination pathways of glutamine oxidation in Ehrlich ascites carcinoma (EAC) and AS 30D hepatoma revealed that hepatoma cells, unlike EAC, produce very little aspartate. This cannot be explained by differences in the activity of glutamine-metabolizing enzymes. Also, the mitochondria from the hepatoma respired at a similar rate to EAC mitochondria with glutamine as sole substrate producing substantial amounts of aspartate. Unlike their isolated mitochondria, intact hepatoma cells showed a very low rate of glutamine oxidation. Compared with EAC, the rate of L-[U-¹⁴C]glutamine consumption by AS 30D hepatoma cells was much lower, with insignificant production of ¹⁴C-labelled aspartate and CO₂. This suggested that the glutamine-transporting system in the hepatoma cell plasma membrane had a very low activity. Isolated hepatoma mitochondria produced 3 times more pyruvate from malate than did EAC mitochondria, indicating a higher activity of NAD(P)-dependent malic enzyme. We postulate that an active malic enzyme may suppress the synthesis of aspartate in hepatoma cells, but further evidence is needed to confirm this assumption.

INTRODUCTION

Although amino acids are primarily precursors for biosynthetic reactions, they are also used as a source of energy. It has been estimated that in man on an average diet approx. 100 g of amino acids daily undergoes oxidative degradation. Among all amino acids, glutamine, glutamate, aspartate and alanine are the most important for cell energetics [1]. A substantial part of their metabolism occurs in mitochondria, where key enzymes such as glutaminase, glutamate-oxaloacetate transaminase (GOT), glutamate-pyruvate transaminase (GPT) and glutamate dehydrogenase (GDH) are located. These amino acids are directly coupled to the citric acid cycle, which is the ultimate pathway for oxidation of their carbon skeleton. Glutamine is the most important precursor of glutamate, aspartate and alanine taken up from blood by cells. It is readily transported across the plasma membrane and the mitochondrial inner membrane on a specific glutamine carrier [1]. Owing to the mitochondrial location of glutaminase, glutamate is generated inside the mitochondria where it can be oxidized via the GOT, GPT and GDH pathways. There is a high rate of glutamine oxidation in cells which possess an active mitochondrial glutaminase. This holds especially for the mitochondria of kidney tubules, enterocytes [2], lymphocytes [3] and some other normal cells. It is interesting, however, that the mitochondria from malignant tumours and rapidly dividing cells have a high oxidative degradation of glutamine, so that glutamine becomes an important energy fuel [4]. Further, tumours induce proteolysis in skeletal muscle [5]. This results in the generation of a significant amount of glutamine, which is rapidly oxidized by malignant cells. For that reason, the understanding of the regulation and function of glutamine oxidation is of great physiological and pathophysiological importance. Although it is generally accepted that there is a high rate of glutamine oxidation in rapidly dividing cells, the problem of regulation and function of this oxidation is poorly understood. It has been proposed that the function of glutamine oxidation is primarily to provide precursors for biosynthetic reactions (e.g. synthesis of purines and pyrimidines) rather than to provide energy fuel [6]. It is also suggested that increased glutamine

oxidation results from an imbalance in citrate oxidation in tumour mitochondria, especially those from hepatoma cells [7].

The aim of our investigation was to throw more light on the problem of regulation and function of glutamine oxidation in malignant cells. It was proposed that an important role of glutamine oxidation is the production of aspartate, which can be used for regeneration of the adenine nucleotide pool through the purine nucleotide cycle. Experiments with Ehrlich ascites carcinoma (EAC) cells supported this proposal [8]. Also, it was shown that the cells of AS 30D hepatoma do not use this pathway for the resynthesis of adenylates [9], and that they have a very low rate of synthesis of aspartate. This was the reason to undertake a detailed study of the pathways of glutamine oxidation, with the question why AS 30D hepatoma compared with EAC has much lower capability for synthesis of aspartate.

MATERIALS AND METHODS

EAC and AS 30D hepatoma were grown in the abdominal cavities of Swiss mice and Sprague-Dawley rats respectively. Both tumours are rapidly growing and poorly differentiated, with very high glycolytic capacity [10]. The cells were harvested 7–10 days after transplantation, separated from ascites fluid and washed twice in Krebs-Ringer phosphate solution, pH 7.4 [8]. All experiments were carried out *in vitro*. The cells were incubated in the same solution with addition of 20 mM-Mops/Tris in order to increase buffering capacity.

The mitochondria were isolated as described by Moreadith & Fiscum [11], by using digitonin to destabilize the plasma membrane. They were incubated in the standard incubation medium, containing 100 mM-KCl, 10 mM-Tris/HCl, 20 mM-Mops/Tris, 10 mM-phosphate (K⁺ salt), 2 mM-MgCl₂, 2 mM-EDTA and bovine serum albumin (1.5 mg/ml), final pH 7.4.

Measurements of metabolites and enzyme activities were performed by standard enzymic methods [12]. Oxygen uptake was determined by an oxygen electrode, whereas protein was measured by the biuret reaction.

The rate of glutamine consumption and metabolism by the

Abbreviations used: EAC, Ehrlich ascites carcinoma; GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvate transaminase; GDH, glutamate dehydrogenase.

Fig. 1. Production of aspartate, glutamate and alanine in EAC and AS 30D hepatoma cells incubated with glutamine

The cells were incubated in Krebs–Ringer phosphate medium containing 20 mM-Mops/Tris, pH 7.4. Additions: 10 mM-glutamine, 5 mM-pyruvate and 130 mg of cells/ml. ○, △, AS 30D hepatoma cells; ●, ▲, EAC cells; ○, ●, Glutamine; △, ▲, glutamine plus pyruvate.

cells was determined by measuring the uptake of L-[U-¹⁴C]glutamine and the generation of ¹⁴C-labelled glutamine, aspartate and CO₂ as described elsewhere [13]. Radioactivity was measured with a Packard liquid-scintillation counter (model 300).

The measurements are expressed in nmol/mg wet wt. of cells or in nmol/mg of mitochondrial protein. Results are from 3–4 separate experiments designed to be as nearly identical as possible.

RESULTS AND DISCUSSION

Formation of aspartate, glutamate and alanine in the cells of EAC and AS 30D hepatoma incubated with glutamine

Parallel incubation of the cells in the same incubation medium in the presence of glutamine with or without addition of pyruvate showed that there is virtually no production of aspartate in hepatoma cells (Fig. 1). In EAC cells this production is high, so that a large amount of aspartate accumulates. Addition of pyruvate strongly decreased the formation of aspartate in EAC, but its accumulation was still significant. The level of endogenous aspartate was not significantly different in these two kinds of tumour cells. However, the concentration of glutamate was markedly higher in hepatoma cells.

There was no production of alanine from glutamine in EAC in the absence of pyruvate, but addition of pyruvate markedly stimulated alanine production. In hepatoma cells there is a low production of alanine from glutamine with or without addition of pyruvate, although alanine production was slightly higher in the presence of pyruvate.

Activity of glutamine-metabolizing enzymes

The very low or insignificant production of aspartate in the AS 30D hepatoma cells suggested that glutamine oxidation in these cells is slow. One of the main reasons may be low activity of glutamine-metabolizing enzymes. However, the data presented in Table 1 do not support this assumption: the activity of glutaminase is approx. 2-fold lower in hepatoma cells (see also

Table 1. Activity of the enzymes of glutamine metabolism in isolated mitochondria and cytosol of EAC and AS 30D hepatoma

Subcellular fractions were obtained by the digitonin method. For measurement of phosphate-dependent glutaminase (PDG) activity the incubation medium contained 100 mM-phosphate (K⁺ salt), pH 8, whereas glutamate, as the product of the reaction, was measured enzymically. GOT, GPT and GDH activities were estimated by standard methods [12].

Cell fraction	Activities (nmol/min per mg of protein)			
	PDG	GOT	GPT	GDH
EAC				
Mitochondria	46	1179	63	173
Cytosol	–	44	13	–
AS 30D hepatoma				
Mitochondria	19	1736	39	100
Cytosol	–	84	8	–

Table 2), but the activity of GOT is even higher than that of the EAC enzyme. Also, the rate of respiration of isolated mitochondria in the presence of glutamine as sole substrate is not much lower than that of EAC mitochondria, indicating that the hepatoma contains an active glutaminase. The high level of glutamate in hepatoma cells strongly supports the conclusion that availability of glutamate cannot be the reason for low production of aspartate.

Metabolism of glutamine in tumour mitochondria

Table 2 shows data which indicate that the production of glutamate is approx. 2-fold higher and that of aspartate is about 3-fold higher in the mitochondria from EAC cells than in mitochondria from hepatoma cells. That aspartate formation is not limited simply by glutaminase activity is indicated by the data regarding the synthesis of aspartate in the presence of

Table 2. Metabolism of glutamine, pyruvate and malate in isolated mitochondria from EAC and AS 30D hepatoma cells

The mitochondria were incubated in the standard medium containing 4.8 mM-glyoxylate (Na⁺salt). Additions: 11 mM-glutamine, 1 mM-ADP, 5.5 mM-pyruvate, 11 mM-malate, 11 mM-glutamate and the mitochondria (4 mg of protein/ml). Samples were taken at 0, 15 and 30 min for subsequent determination of metabolites. The measurements are expressed in nmol of metabolite/15 min per mg of protein. Where production of pyruvate from malate was investigated, the medium contained 1 mM-arsenite instead of glyoxylate. Abbreviations: ND, not detectable; –, not measured.

Substrates	EAC					AS 30D hepatoma				
	Glu	Asp	Ala	Citrate	Pyruvate	Glu	Asp	Ala	Citrate	Pyruvate
Glutamine	154.6	91.9	5.8	ND	–	76.2	34.4	ND	ND	–
Glutamine + pyruvate		14.8	57.5	12.3	–		21.8	21.0	ND	–
Glutamate + malate		153.2	ND	–	–		84.1	14.6	–	–
Glutamate + pyruvate		21.5	53.9	10.6	–		24.7	10.7	ND	–
Malate + pyruvate				38.3	–				36.2	–
Malate					21.0					61.0

glutamate plus malate, the condition when maximal synthesis is expected. However, even under these conditions aspartate production is twice as much in EAC mitochondria as in hepatoma mitochondria.

The addition of pyruvate resulted in a strong suppression of aspartate production from glutamine in both kinds of mitochondria, with stimulation of alanine synthesis, which was 3-fold higher in EAC mitochondria. Similar results were obtained in the presence of glutamate instead of glutamine as substrate.

Since NAD(P)-dependent malic enzyme, which is usually found in the mitochondria from malignant cells [14,15], produces pyruvate from malate, it was possible that its activity could have a significant influence on the pathways of glutamine oxidation. It is expected that the production of pyruvate should decrease the oxidation through the aspartate aminotransferase pathway and increase the synthesis of alanine or citrate instead of aspartate. It was found that the production of pyruvate from malate in the presence of arsenite was approx. 3-fold higher in hepatoma than in EAC mitochondria (Table 2). We did not find a significant difference in the production of citrate from malate plus pyruvate in the presence of glyoxylate, which is an inhibitor of the citric acid cycle at the level of isocitrate dehydrogenase. Moreadith & Lehninger [16] reported that isolated mitochondria from AS 30D hepatoma produce alanine and citrate, but very little aspartate. They pointed out that hepatoma mitochondria produce pyruvate only from extramitochondrial malate.

Rates of respiration of isolated tumour mitochondria with different substrates

Although the rate of respiration of hepatoma mitochondria in the presence of glutamine is slightly slower than that of EAC mitochondria, it is faster than with glutamate as substrate. This suggests that glutaminase activity is not the rate-limiting factor. This is supported by the finding which shows that an increase in glutamine concentration increases glutaminase activity, but not the rate of respiration [17]. It was found that hepatoma mitochondria respired at a maximal rate in the presence of less than 1 mM-glutamine, whereas the K_m of glutaminase for the substrate in intact organelles is 2.8 mM.

Contrary to the data reported from other laboratories [7], the mitochondria isolated from EAC and AS 30D hepatoma do respire in the presence of pyruvate, and the rate of respiration was not markedly slower than with glutamine as substrate. Also, the mitochondria respire with citrate. The respiration of EAC mitochondria is quite slow, but the mitochondria from hepatoma cells respire much faster with citrate than with glutamine, which is also in contrast with the data reported by Coleman & Parlo [7].

Table 3. Rate of respiration of isolated mitochondria from EAC and AS 30D hepatoma cells in the presence of various substrates

Incubations were carried out in the standard incubation medium containing 1.5 mM-glutamine, 1.5 mM-glutamate, 1.5 mM-pyruvate, 0.3 mM-malate, 3.8 mM-citrate, 0.6 mM-ADP, 10 mM-glyoxylate and the mitochondria (1.2 mg of protein/ml).

Substrates	Rate of respiration (ng-atoms of oxygen/ min per mg of protein)	
	EAC	AS 30D hepatoma
Glutamine	33.9	25.0
Pyruvate	32.9	32.5
Glutamine + pyruvate	130.7	70.0
Glutamine + malate	45.4	32.5
Glutamine + glutamate	73.3	45.8
Glutamate	28.6	23.3
Citrate	15.1	53.0
Glyoxylate + pyruvate	7.0	0
Glyoxylate + pyruvate + glutamine	46.7	32.5

The fastest rate of respiration was achieved with glutamine plus pyruvate, because pyruvate provides a sufficient amount of acetyl-CoA to react with the oxaloacetate generated by glutamine oxidation. This is supported by the finding that respiration is equally fast with pyruvate plus malate as with glutamine plus pyruvate.

Table 3 shows that glyoxylate inhibited respiration with pyruvate, whereas the addition of glutamine abolished this inhibition, indicating that glutamine (or glutamate) oxidation occurs through the 'truncated Krebs cycle' [7]. This could be one reason why glutamine is a useful energy fuel for some kinds of tumour cells.

Consumption and metabolism of L-[U-¹⁴C]glutamine

Since the data concerning the AS 30D hepatoma suggest a very slow rate of glutamine oxidation, the question arose of the magnitude of the rate of its consumption by the cells. Experiments with ¹⁴C-labelled glutamine showed clearly that there is a much higher rate of glutamine utilization by EAC compared with AS 30D hepatoma cells (Fig. 2). For EAC, these data are in agreement with our previous observation [13]. Also, they indicate that in this case approx. 60 % of radioactivity from [U-¹⁴C]glutamine was recovered as ¹⁴C-labelled glutamate, aspartate

Fig. 2. Consumption of L-[U- ^{14}C]glutamine and production of ^{14}C -labelled glutamate, aspartate and CO_2 by EAC and AS 30D hepatoma cells

The cells (140–150 mg wet wt. in 1 ml final volume) were incubated in 25 ml Erlenmeyer flasks. The central well contained 10% Hyamine as a trap for $^{14}\text{CO}_2$. Incubations were carried out aerobically in the presence of L-[U- ^{14}C]glutamine (5 mM for EAC or 2 mM for hepatoma cells; 6.6 kBq/ml). The incubation was stopped by addition of 0.3 ml of trichloroacetic acid (20%, w/v) and the flasks were kept closed overnight at 0 °C to trap all $^{14}\text{CO}_2$. The trichloroacetic acid extract was used for separation of radioactive glutamine, glutamate and aspartate on an ion-exchange column (7 cm $\times$ 0.6 cm) of Dowex 1X8 (acetate form). Because glutamine was eluted in the same fraction with other neutral substances, this fraction was heated for 2 h with 2 M-HCl at 100 °C. Rechromatography of this material revealed that there was no significant radioactivity in the glutamine fraction, since it was hydrolysed to glutamate.

and CO_2 , whereas production of $^{14}\text{CO}_2$ and [^{14}C]aspartate was insignificant in hepatoma cells. An increase in glutamine concentration (with the same specific radioactivity) up to 40 mM did not result in a significant increase in its consumption or production of ^{14}C -labelled aspartate or CO_2 . It is therefore possible that the very low rate of glutamine consumption by hepatoma cells results from a low activity of the glutamine-transporting system in the plasma membrane of the cells, but this assumption needs additional experimental support.

Production of citrate from pyruvate or glutamine in the cells of EAC and AS 30D hepatoma, and effect of glyoxylate

It was proposed that there is an imbalance in citrate oxidation in tumour mitochondria owing to a rapid efflux of citrate into the cytosol, where it can be used for intensive synthesis of cholesterol [7]. In order to overcome this difficulty, mitochondria increase glutamine oxidation via the 'truncated Krebs cycle'. In accordance with this proposal, it can be assumed that the decrease

Table 4. Effect of glyoxylate on the concentration of citrate in EAC and AS 30D hepatoma cells in the presence of pyruvate or glutamine

The cells were incubated in Krebs–Ringer phosphate medium with addition of 20 mM-Mops/Tris, pH 7.4. Additions: 10 mM-pyruvate, 10 mM-glutamine, 5.6 mM-glyoxylate and 130 mg of cells/ml.

Experimental conditions	Citrate (nmol/mg of cells)	
	EAC	AS 30D hepatoma
Control	0.52	0.93
Pyruvate	0.55	0.59
Glyoxylate + pyruvate	3.31	2.81
Glyoxylate + glutamine	2.17	0.53

of aspartate synthesis in AS 30D hepatoma cells results from an increased production of citrate. However, the data presented in Table 4 (and in Tables 2 and 3) do not support this assumption. Hepatoma cells and their isolated mitochondria do not have a higher capacity for citrate synthesis than do EAC cells or mitochondria. Table 4 shows that there is no accumulation of citrate in either hepatoma or EAC cells in the presence of pyruvate unless glyoxylate was added. Further, EAC accumulate citrate in the presence of glutamine as sole substrate, whereas hepatoma cells do not.

All these results indicate that there is a significant difference in glutamine oxidation between Ehrlich ascites carcinoma and AS 30D hepatoma cells. This is especially true for glutamine metabolism in the intact cells where a large quantitative difference was found. Not only glutamine oxidation but also its hydrolysis in intact hepatoma cells is very low compared with the same activities in isolated mitochondria. This finding indicates that, at least in this case, it is not easy to extrapolate the data obtained with isolated mitochondria to the situation in intact cells. It is not possible to draw this conclusion from the data reported by other laboratories. For the AS 30D hepatoma, Moreadith & Lehninger [16] reported a high rate of glutamine oxidation in isolated mitochondria, but they did not study intact cells. Kelleher and co-workers [18] studied glutamine metabolism in intact AS 30D hepatoma cells, and concluded that $^{14}\text{CO}_2$ data support the hypothesis that the cells possess a capacity for conversion of extracellular glutamine into citric acid-cycle intermediates that is quantitatively significant relative to citric acid-cycle flux. However, since all the results were obtained by measuring radioactivity of metabolites and expressed in d.p.m., it is difficult to calculate the true rate of glutamine metabolism in the cells.

The high production of aspartate in EAC can be correlated with the regeneration of the pool of adenine nucleotides via the purine nucleotide cycle. Hepatoma cells do not use the same mechanism for resynthesis of AMP [9]. This suggests that in some tumour cell lines a high activity of the aspartate aminotransferase pathway might be important for resynthesis of adenylates and, therefore, for energy status of the cells.

The main function of the alanine aminotransferase pathway in tumours is probably the removal of excess of pyruvate generated by glycolysis. Although production of pyruvate by malic enzyme is much lower than pyruvate production by glycolysis, its formation inside the mitochondria, where it is separated and protected from lactate dehydrogenase, might be a very efficient mechanism for inhibition of mitochondrial GOT and stimulation of alanine or citrate formation.

Although the mitochondria from both tumours contain NAD(P)-dependent malic enzyme [14–16], the present data and those of Kelleher *et al.* [18] do not suggest that this enzyme has

an important role in the complete oxidation of glutamine in CO₂. There is no doubt that it could have a significant influence on the control of glutamine oxidation in terms of inhibiting the aspartate aminotransferase pathway and channelling the glutamine carbon skeleton into alanine or citrate. This holds especially if an increase in the activity of malic enzyme is accompanied by a decrease in the activity of mitochondrial malate dehydrogenase, as in proliferating fibroblasts [19].

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REFERENCES

1. Kovačević, Z. & McGivan, J. D. (1983) *Physiol. Rev.* **63**, 547–605
2. Windmueller, H. G. & Speath, A. E. (1980) *J. Biol. Chem.* **255**, 107–112
3. Ardawi, M. S. M. & Newsholme, E. A. (1985) *Essays Biochem.* **21**, 1–44
4. Kovačević, Z. & Morris, H. P. (1972) *Cancer Res.* **32**, 326–333
5. Rivera, S., Azcoñ-Bieto, J., López-Soriano, F. J., Miralpeix, M. & Argilés, J. M. (1988) *Biochem. J.* **249**, 443–449
6. Newsholme, E. A., Crabtree, B. & Ardawi, M. S. M. (1985) *Biosci. Rep.* **5**, 393–400
7. Coleman, P. S. & Parlo, R. A. (1984) *Ann. N. Y. Acad. Sci.* **435**, 129–132
8. Kovačević, Z., Jeranče, D. & Brkljač, O. (1988) *Biochem. J.* **252**, 381–386
9. Kovačević, Z., Brkljač, O. & Jeranče, D. (1991) *Biochem. J.* **273**, 277–281
10. Smith, D., Walborg, E. F. & Chang, J. P. (1970) *Cancer Res.* **30**, 2306–2309
11. Moreadith, R. W. & Fiskum, G. (1984) *Anal. Biochem.* **137**, 360–367
12. Bergmeyer, H. U. (ed.) (1984) *Methods of Enzymatic Analysis*, 3rd edn., Verlag Chemie, Weinheim
13. Kovačević, Z. (1972) *Eur. J. Biochem.* **25**, 372–378
14. Sauer, L. A., Dauchy, R. T., Nagel, W. C. & Morris, H. P. (1980) *J. Biol. Chem.* **255**, 3844–3848
15. McKeehan, W. L. (1986) in *Carbohydrate Metabolism in Cultured Cells* (Morgan, M. J., ed.), pp. 111–150, Plenum, New York
16. Moreadith, R. W. & Lehninger, A. L. (1984) *J. Biol. Chem.* **259**, 6215–6221
17. Kovačević, Z. (1971) *Biochem. J.* **125**, 757–763
18. Kelleher, J. K., Bryan, B. M., Mallet, R. T., Holleran, A. L., Murphy, A. N. & Fiskum, G. (1987) *Biochem. J.* **246**, 633–639
19. McKeehan, W. L. & McKeehan, K. A. (1982) *J. Cell. Physiol.* **110**, 142–148

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