Properties and Intracellular Localization of Ehrlich Ascites Tumor Cell Glutaminase[1]

Zoran Kovačevic

Department of Biochemistry, Medical Faculty, 21000 Novi Sad, Yugoslavia

SUMMARY

The characteristics and intracellular localization of Ehrlich ascites tumor cell glutaminase were investigated. It was found that these cells possess only the phosphate-dependent glutaminase isoenzyme, which requires 50 mM inorganic phosphate for maximum activation. The K_m value of the enzyme for glutamine was 4.5 mM, and the optimum pH was between 8.0 and 8.5. Differential centrifugation of the cell homogenate revealed that glutaminase strictly follows the distribution of glutamate dehydrogenase, indicating that this enzyme has an exclusively mitochondrial localization. Disintegration of the mitochondria led to the complete inactivation of glutaminase, but the addition of borate protected the enzyme when detergents were used. The treatment of the mitochondria with digitonin followed by centrifugation showed that glutaminase is located in the mitochondrial matrix.

INTRODUCTION

It has been well documented that phosphate-dependent glutaminase isoenzyme (L-glutamine amidohydrolase; EC 3.5.1.2) has mainly mitochondrial localization in some normal cells (rat liver and kidney) (3, 7, 11). Our previous investigation showed that isolated mitochondria from Ehrlich ascites tumor cells use glutamine as a very good substrate (9, 12). This suggested the mitochondrial localization of glutaminase in these cells, in view of previous evidence (9) that glutamine transaminase could not be responsible for the ability of the mitochondria to respire in the presence of glutamine, because the respiration was not inhibited by a transaminase inhibitor. However, we could not find experimental evidence of the exact intracellular localization of the enzyme in Ehrlich ascites cells. Therefore, the purpose of this study was to determine the intracellular localization of glutaminase, with the hope that this would clarify the role of glutamine in the oxidative metabolism of the tumor cells and the origin of the intramitochondrial-reducing equivalents.

MATERIALS AND METHODS

Cell Homogenization and Fractionation of Subcellular Particles by Means of Differential Centrifugation. Ehrlich ascites tumor cells were maintained by i.p. transplantation in

white Swiss mice and were harvested 8 to 10 days later. The cells were separated from ascites fluid by centrifugation and washed several times in order to remove erythrocytes. The packed cells were diluted with redistilled water (ratio, 1:3) and rapidly transferred to a Potter-Elvehjem homogenizer. The cells were mixed by 8 passes (50 sec). The tonicity was then rapidly raised to 0.25 M by adding 2 M sucrose, and the contents were thoroughly mixed. The cells were sedimented and resuspended in the isolation medium containing 0.25 M sucrose, 5 mM Tris-Cl, and 1 mM ethanedioxybis(ethylamine)-tetraacetate; the final pH was 7.4. After vigorous homogenization, the material was fractionated by means of a Spinco Model L preparative ultracentrifuge, and the following fractions were obtained. Nuclear Fraction N (7 min at 550 X g) was represented by the nuclei, whole cells, and debris. This fraction was rehomogenized 2 times and the supernatants were combined to make cytoplasmic Extract E. The cytoplasmic extract was centrifuged to obtain mitochondrial Fraction M (10 min at 8,830 X g) and microsomal Fraction P (60 min at 105,000 X g). The mitochondrial fraction was washed twice and the washings were combined with the 1st supernatant. The final supernatant, obtained after the sedimentation of microsomes, represented the soluble or cytoplasmic Fraction S. In each case the sediments were resuspended in the isolation medium. The whole experiment was repeated twice with different batches of tumor cells, and the means of the 3 determinations were calculated.

Enzyme Assays. These were carried out as described elsewhere (11), except that the incubation medium for the glutaminase assay was of the following composition: 50 mM P_i (K^+ salt), 50 mM Tris-Cl, and 20 mM glutamine. The final pH was 8.0, and the temperature was 30°. Blank values obtained with the complete reaction mixture without incubation were always subtracted. In the case of glutaminase, all values were corrected for nonenzymatic hydrolysis of glutamine. All enzyme assays were done in triplicate and the means of the 3 determinations were recorded. The results were consistent and reproducible.

RNA Estimation. Since the activity of glucose 6-phosphatase could not be detected in these cells, RNA was used as the marker for the microsomal fraction. It was measured colorimetrically, as described elsewhere (14).

Cytochrome c oxidase was assayed polarographically (11) by measuring the oxygen consumption with a Clark electrode at 30° in an assay system containing Tris-Cl (20 mM), sodium ascorbate (4 mM), cytochrome c (1 mg), and digitonin (0.5 mg/mg protein) Merck, Darmstadt, Germany). The final volume was 5 ml, and the final pH was 7.4.

Protein was determined by a modified biuret method (2), and deoxycholate was added to solubilize the particles.

RESULTS

Kinetic Studies on Glutaminase. Although it is known that normal tissues (*e.g.*, liver, kidney, brain) possess 2 types of glutaminase isoenzymes (6), one activated by phosphate (phosphate-dependent glutaminase) and the other not requiring this anion for activation (phosphate-independent glutaminase), we could detect no significant activity of the latter isoenzyme, even in the presence of maleate. Therefore, the so-called phosphate-dependent glutaminase was the isoenzyme chosen for this study.

The kinetic constants of glutaminase, the K_m and V_{max} for glutamine, were estimated by the graphical method of Lineweaver and Burk. The K_m of glutaminase for glutamine was determined to be about 4.5 mM and the V_{max} was 0.176 μmole glutamine per min per mg protein. The incubation medium was the same as described in "Materials and Methods" except that the glutamine concentration was varied (from 5 to 40 mM).

Chart 1 shows that Ehrlich ascites cell glutaminase is strongly activated by inorganic phosphate, the K_a for phosphate being 15.6 mM. For maximum activity, 50 mM phosphate was required.

By plotting the pH values of the incubation medium *versus* corresponding activities of glutaminase, a pH optimum of between 8.0 and 8.5 was obtained (Chart 2).

All the enzyme assays were done with isolated mitochondria that were kept at $-15°$ overnight. It should be noted that repeated freezing and thawing does not release (and does not

inactivate) the enzyme from the mitochondria.

Effect of Sonic Treatment and Detergents on the Activity of Glutaminase. When the mitochondria were subjected to ultrasonic disintegration (5 X 15 sec, 3 μm; MSE sonicator), complete inactivation of glutaminase was obtained despite the fact the temperature was kept steady near $0°$ and that the mitochondrial suspension was very thick. The same results were obtained by adding 0.1% Triton X-100 (Table 1). Borate protected the enzyme in the case of the detergent but also inhibited the hydrolytic reaction in intact mitochondria about 25%. It is possible that the lower activity observed in the detergent-treated mitochondria compared to intact mitochondria resulted from the stronger inhibition by borate and not from solubilization of the enzyme. No way was found to protect glutaminase during sonic treatment. The addition of glutamine without borate before the detergent treatment protected the enzyme about 50%.

Intracellular Localization of Glutaminase. The distribution of glutaminase, the reference enzymes, and RNA found after differential centrifugation is shown graphically in Chart 3 in

Chart 2. The pH dependence of glutaminase activity. The corresponding pH values of the incubation medium (described in "Materials and Methods") were adjusted by Tris or HCl.
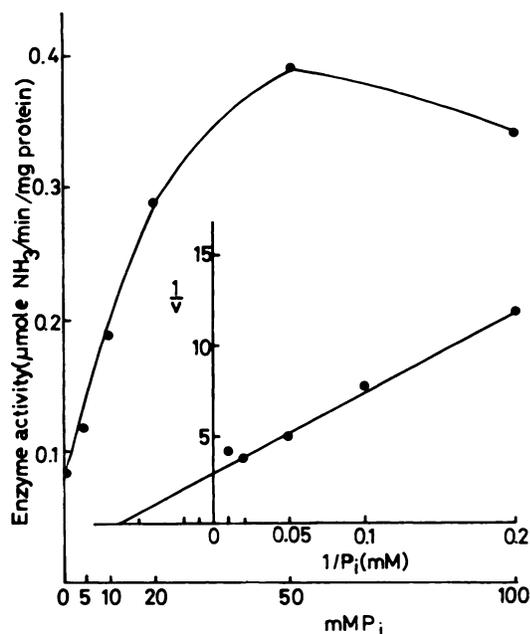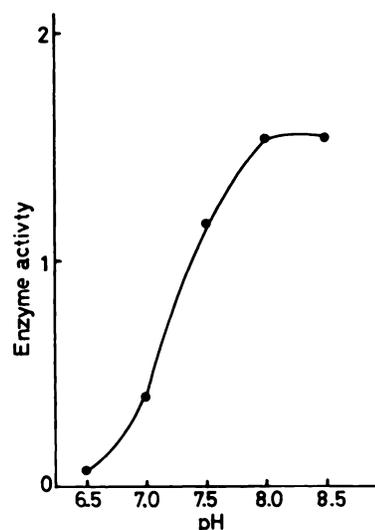
Chart 1. Influence of P_i concentration on glutaminase activity of Ehrlich ascites cells. The incubation medium was the same as described in "Materials and Methods" except that the P_i concentration was varied.

Table 1

Effect of sonic treatment and of Triton X-100 on the activity of glutaminase in the presence and absence of borate

The mitochondria were washed with Tris-Cl (130 mM, pH 7.4) and then suspended in the medium containing 20 mM P_i and 20 mM borate, pH 8.0. Before sonic treatment or treatment with Triton X-100, the mitochondria were preincubated in this medium for 30 min at 37°. The enzyme assay was carried out in the incubation medium described in "Material and Methods" except that, in this case, 20 mM borate was present.

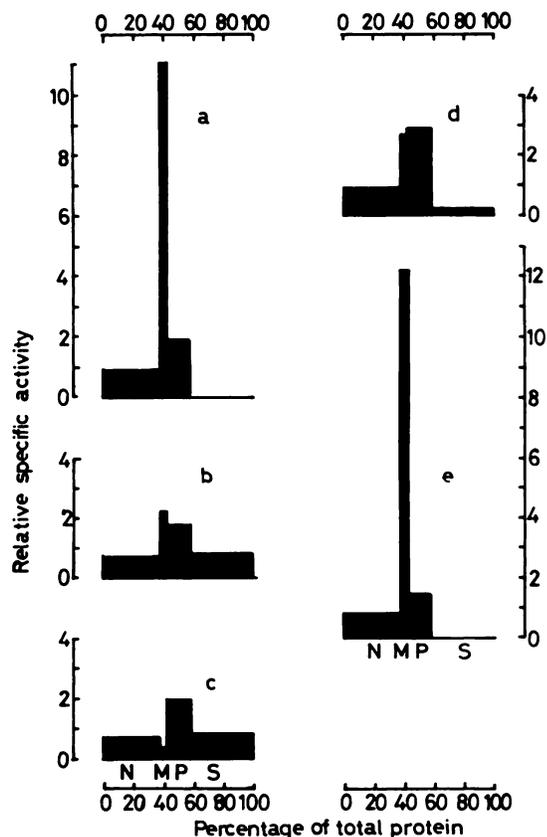| Treatment of mitochondria	% activity of glutaminase	
	Without borate	With borate
Control	100	100
Sonic disintegration	5	5
Triton X-100	7	78
Triton X-100 (added after glutamine)	44	

Chart 3. Distribution patterns of enzymes and RNA in Ehrlich ascites tumor cells: *a*, glutamate dehydrogenase; *b*, acid phosphatase; *c*, RNA; *d*, NADH-cytochrome *c* reductase (rotenone insensitive); *e*, glutaminase. *Abscissa*, relative protein concentration of fractions— nuclear (*N*), mitochondrial (*M*), microsomal (*P*), and soluble (*S*). *Ordinate*, relative specific activity of fractions. Recovery: protein, 100.0%; glutamate dehydrogenase, 102.0%; acid phosphatase, 91.9%; RNA (88.7%), NADH-cytochrome *c* reductase (93.2%); glutaminase 95.8%. The sum of the values obtained separately on the nuclei and cytoplasmic extract was taken as representative of the whole cells (N + E = 100%).

the manner proposed by De Duve *et al.* (1).

In all cases the nuclear fraction contained about 30% of the activity of marker enzymes studied, the same was found for glutaminase. This could be explained by contamination of the nuclear fraction and the presence of unbroken cells. Glutamate dehydrogenase, acid phosphatase, RNA, and rotenone-insensitive NADH-cytochrome *c* reductase were the markers for mitochondria, lysosomes, microsomes, and the outer mitochondrial membrane and microsomes, respectively. It can be seen from Chart 3 that the distribution pattern of glutaminase is almost identical to that of glutamate dehydrogenase.

Separation of the Mitochondrial Membranes from the Matrix by Centrifugation after Treatment with Digitonin. In order to determine submitochondrial localization of glutaminase, the mitochondria were treated with digitonin according to the method of Schnaitman *et al.* (15). The mitochondria were first washed with Tris-Cl (130 mM, pH 7.4) and then mixed with digitonin (0.5 mg digitonin per mg protein). The digitonin solution was prepared by sonic dispersion in a 10 mg/ml concentration of phosphate (20 mM)-borate (20 mM)

buffer, pH 8. The mitochondria mixed with digitonin were kept on ice for 20 min, then were diluted with the same phosphate-borate buffer and centrifuged 60 min at 105,000 X *g*. The supernatant was decanted and the pellet was resuspended in the same volume of buffer. This material and the suspension of the mitochondria in the same buffer without digitonin (control) were used to estimate the activity of glutaminase, glutamate dehydrogenase, and cytochrome *c* oxidase. Glutaminase was assayed in the presence of 20 mM borate. Table 2 shows that glutaminase closely follows the distribution of glutamate dehydrogenase, which was used as the marker for the mitochondrial matrix.

Inhibition of Glutaminase by Glutamate in Intact and Triton-treated Mitochondria. As a product of the enzymatic reaction, glutamate does not inhibit glutaminase in the intact mitochondria, up to a concentration of 10 mM. In higher concentrations, glutamate inhibits this enzyme (Chart 4). The same chart shows that glutamate inhibits glutaminase much more strongly in the preparation of Triton-treated mitochondria.
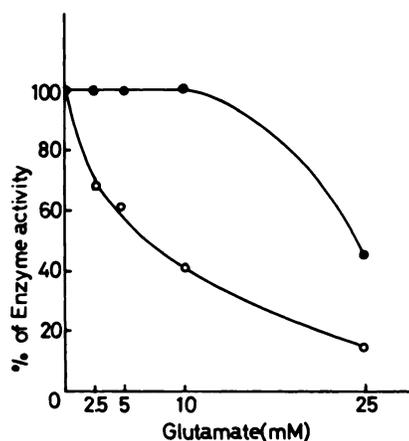

Chart 4. Inhibition of glutaminase activity by glutamate in intact (•), and Triton-treated (o) mitochondria. In both cases, the same incubation medium was used as described in Table 1. Before the addition of glutamine, the mitochondria were preincubated for 15 min. Glutamate was added together with glutamine. In this experiment, freshly prepared, coupled mitochondria were used.

Table 2

Distribution of protein and enzyme activities in the mitochondrial membrane and the matrix fraction

The mitochondria were treated with digitonin, and the fractions were separated by centrifugation.

Fractions	% values			
	Protein	Cytochrome oxidase	Glutamate dehydro-genase	Gluta-minase
Total[a]	100	100	100	100
Pellet	53	82	18	20
Soluble	51	18	64	65
Recovery	104	100	82	85

[a] Mitochondria were suspended in phosphate-borate buffer without digitonin.

DISCUSSION

Two glutaminase isoenzymes were found in the cells of the rat kidney, liver, and brain (6). However, no phosphate-independent glutaminase activity was detected in the ascites cells studied. The phosphate-activated glutaminase was the only isoenzyme found in these cells. Kinetic analyses have shown that for optimum activity, this enzyme needs a high concentration of phosphate and an incubation medium with a very high pH. In this respect, it is similar to the kidney type of phosphate-dependent glutaminase (4). The enzyme needs about 4.5 mM glutamine for the half-maximum activity. In connection with this, it is interesting that isolated mitochondria from these cells require a much lower concentration of glutamine (about 0.2 mM) to achieve the maximum rate of respiration in the presence of this substrate. Apparently, the activity of glutaminase is not the rate-limiting step in this case.

It should be noted that glutaminase from Ehrlich ascites cells, like phosphate-activated glutaminase from other sources (rat liver and kidney), is highly sensitive to disintegration of mitochondria. The addition of borate protects the enzyme from inactivation by detergents. However, in the case of ultrasonic disintegration of mitochondria, the protective effect of borate is absent. Repeated freezing and thawing of the mitochondria does not inactivate glutaminase, but there is no significant release of the enzyme from the organelles. The same was found for glutamate dehydrogenase.

Differential centrifugation of the cell homogenate showed that glutaminase strictly follows glutamate dehydrogenase as the marker enzyme for mitochondria. This finding strongly suggests that glutaminase is exclusively a mitochondrial enzyme. Disintegration of the mitochondria by treatment with digitonin, and separation of the mitochondrial membranes from the mitochondrial matrix by centrifugation, revealed that glutaminase follows glutamate dehydrogenase again but not cytochrome oxidase. Since the distribution of a marker enzyme for the intermembrane space was not followed, the above finding is insufficient to confirm the matrix location of the enzyme studied. However, it was found that glutamate inhibits glutaminase much more strongly in the preparation of the Triton-treated mitochondria than in the preparation of intact organelles. In addition, other data suggest that during the activity of glutaminase, glutamate is produced inside the mitochondria (12). All these findings indicate that the enzyme is probably located in the mitochondrial matrix.

It has been shown that glutaminase plays a significant role in the metabolism of tumors because it is quantitatively proportional to growth rate and morphology of neoplasms (5, 8). The strictly mitochondrial localization of glutaminase, which is one of the major enzymes in glutamine metabolism, indicates once again that this metabolite is very important for the oxidative and energy metabolism of ascites tumor cells (12). The location of glutaminase in the mitochondrial matrix and rapid intramitochondrial generation of glutamate suggest that the enzyme plays an important role in the production of reducing equivalents inside the mitochondria. This should lead to an increase of the reduced state of intramitochondrial pyridine nucleotides, which is, according to Krebs and Veech (13), generally very important for the oxidative and energy

metabolism of mitochondria. In connection with this it should be noted that the oxidation of glutamate (originating from glutamine) goes mainly through the glutamate dehydrogenase pathway instead of the aspartate transaminase pathway if pyruvate of glucose is present (9). Therefore, in this case, glutamate reduces $NAD(P)^+$ directly through glutamate dehydrogenase reaction. This might be of vital significance for malignant cells in general, since there is no evidence at present concerning the existence of substrate cycles or of any other mechanism that could be responsible for the transfer of reducing equivalents from cytosol to mitochondria in these cells (10, 17).

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