Purification of phosphate-dependent glutaminase from isolated mitochondria of Ehrlich ascites-tumour cells

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Phosphate-dependent glutaminase was purified to homogeneity from isolated mitochondria of Ehrlich ascites-tumour cells. The enzyme had an M_r of 135000 as judged by chromatography on Sephacryl S-300. SDS/polyacrylamide-gel electrophoresis displayed two protein bands, with M_r values of 64000 and 56000. Two major immunoreactive peptides of M_r values of 65000 and 57000 were found by immunoblot analysis using anti-(rat kidney glutaminase) antibodies. The concentration-dependences for both glutamine and phosphate were sigmoidal, with $s_{0.5}$ values of 7.6 mM and 48 mM, and Hill coefficients of 1.5 and 1.6, respectively. The glutaminase pH optimum was 9. The activation energy of the enzymic reaction was 58 kJ/mol. The enzyme showed a high specificity towards glutamine. A possible explanation for the different kinetic behaviour found for purified enzyme and for isolated mitochondria [Kovačević (1974) *Cancer Res.* 34, 3403–3407] should be that a conformational change occurs when the enzyme is extracted from the mitochondrial inner membrane.

INTRODUCTION

Glutamine is described as essential for rapidly dividing cells and tumours (Kovačević & McGivan, 1983). Phosphate-dependent glutaminase activity is correlated with malignancy and growth rate in hepatomas (Linder-Horowitz *et al.*, 1969; Matsuno *et al.*, 1986), fibroblasts (Sevdalian *et al.*, 1980), thymocytes (Brand, 1985) and tumours (Knox *et al.*, 1967, 1969, 1970). In spite of the main role of glutamine in energy and nitrogen metabolism in tumours (Carrascosa *et al.*, 1984; Pérez-Rodríguez *et al.*, 1987; Quesada *et al.*, 1988), studies on phosphate-dependent glutaminase (EC 3.5.1.2) from tumour cells, the first enzyme in glutamine catabolism, are scarce in biochemical literature. Williams & Manson (1958) and Kovačević (1974) described the characteristics of glutaminase, using isolated mitochondria from HeLa and Ehrlich ascites-tumour cells respectively. Huang & Knox (1976) partially purified this enzyme from a mammary carcinoma. Phosphate-dependent glutaminase has been purified from pig kidney (Kvamme *et al.*, 1970) and brain (Svenneby *et al.*, 1973), from rat kidney (Curthoys *et al.*, 1976b) and brain (Haser *et al.*, 1985), and from cow brain (Chiu & Boeker, 1979). The enzyme from rat liver was partially purified by Patel & McGivan (1984), and purification of this glutaminase to homogeneity was reported by Heini *et al.* (1987).

The present paper reports the purification of phosphate-dependent glutaminase from a highly malignant Ehrlich ascites carcinoma, and some molecular and kinetic characteristics of the protein. The method described involves isolation of intact mitochondria, by the procedure of Moreadith & Fiskum (1984), to prevent the inactivation of the enzyme by lysosomal proteinases.

EXPERIMENTAL

Ehrlich ascites cells

A hyperdiploid Lettré strain was maintained in

2-month-old female albino Swiss mice. The cells were harvested from the peritoneal cavity about 10 days after the mice were inoculated with 5×10^6 cells, as described elsewhere (Olavarria *et al.*, 1981). The animals received standard Panlab food with tap water *ad libitum* and were kept at $20 \pm 2^\circ\text{C}$, with light from 08:00 to 20:00 h.

Assay procedure

Phosphate-dependent glutaminase was assayed by the rate of ammonia formation at 25°C , with the glutamate dehydrogenase reaction as an auxiliary system. The rate of NADH oxidation was monitored at 340 nm in a Beckman DU-8B spectrophotometer. The reaction medium contained 100 mM-Tris/HCl, 5 mM-2-oxo-glutarate, 0.2 mM-EDTA, 0.3 mM-NADH, 200 mM-potassium phosphate, 20 mM-glutamine, pH 8, and 48 units of glutamate dehydrogenase/ml in glycerol (Boehringer, Mannheim, Germany). For studies of pH- and temperature-dependence, glutaminase activity was measured by assaying the production of glutamate from glutamine in a stopped assay system. Glutaminase was incubated in 100 mM-Tris/HCl, pH 8, containing 200 mM-potassium phosphate and 20 mM-glutamine. The final volume was 0.5 ml. After 10 min the reaction was stopped by addition of 0.5 ml of 4 M-HCl. The acid extracts were neutralized with 40% (w/v) KOH. Glutamate was assayed as described by Lund (1985). Two control blanks, one with glutamine omitted, the other with glutaminase omitted, were assayed in the same conditions and their values were subtracted. The results obtained by both continuous and stopped assays were consistent. Protein was measured by the Lowry *et al.* (1951) procedure.

Isolation of mitochondria

Ascites-tumour-cell mitochondria were isolated by the method of Moreadith & Fiskum (1984). Rat and mouse kidney mitochondria were isolated essentially as described by Chappell & Hansford (1972) in a

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medium containing 250 mM-sucrose, 10 mM-Tris/HCl, 1 mM-EGTA, 0.5 mM-phenylmethanesulphonyl fluoride, 0.5 mM-benzamidine, pH 7.4.

Purification method

Intact and coupled mitochondria were isolated from 3×10^{10} Ehrlich ascites-tumour cells and suspended at 20–25 mg/ml in buffer A, consisting of 5 mM-Hepes, 210 mM-mannitol, 70 mM-sucrose, 1 mM-EGTA and 20 mM-sodium borate, pH 8. The cytosolic contamination in the mitochondrial fraction was lower than 0.6%, measured as percentage of the total lactate dehydrogenase. No lysosomal contamination, measured as acidic proteinase activity by the method of Turk *et al.* (1984), was detected in the mitochondrial preparations. The isolated mitochondria can be frozen at -30°C for at least 4 months without inactivation of the glutaminase. The mitochondrial suspension was treated for 30 min at 0°C with 0.5% (v/v) Triton X-100, and centrifuged at 105000 *g* for 30 min at 4°C . The mitochondria-free extract was applied to a DEAE-cellulose column (1.8 cm $\times$ 9.0 cm) previously equilibrated with buffer A. The column was washed through with the same medium, and then eluted with a linear potassium phosphate gradient (0–100 mM) in buffer A at a flow rate of 20 ml/h. The total volume of the gradient was 100 ml. Fractions showing glutaminase activity were pooled, concentrated to 1 ml with a CX-30 filter (Millipore, Bedford, MA, U.S.A.) and applied to a Sephacryl S-300 column (1.3 cm $\times$ 35 cm) previously equilibrated with buffer B (50 mM-Tris/HCl, 210 mM-mannitol, 70 mM-sucrose, 1 mM-EGTA, pH 8). The column was eluted with this medium at a flow rate of 10 ml/h; 1 ml fractions were collected.

M_r estimation

The M_r of phosphate-dependent glutaminase was estimated by Sephacryl S-300 chromatography. The marker proteins of defined M_r were catalase (240000), alcohol dehydrogenase (150000), bovine serum albumin (66000) and cytochrome *c* (12400). Blue Dextran was also included as a marker of the void volume. The M_r of the subunit was estimated by electrophoresis in the presence of 1% (w/v) SDS by the method of Laemmli (1970). The marker monomeric proteins of known M_r were bovine serum albumin (66000), egg albumin (45000), carbonic anhydrase (29000) and lactalbumin (14000).

Immunoblot analysis

Immunoblot analysis was carried out essentially as described by Mallison *et al.* (1986). The samples were subjected to SDS/polyacrylamide-gel electrophoresis and then transferred to nitrocellulose. After blocking with 0.3% Tween/PBS solution (137 mM-NaCl, 3 mM-KCl, 8 mM- Na_2HPO_4 , 1 mM- KH_2PO_4 , pH 7.4), containing 5% (w/v) dried milk, the nitrocellulose was incubated for 2 h with a 1:5000 dilution in the same medium of anti-(rat kidney glutaminase) antibodies. It was then incubated for 1 h with a 1:3000 dilution of goat anti-(rabbit IgG)-horseradish peroxidase conjugate (Bio-Rad, Richmond, CA, U.S.A.) in 0.3% Tween/PBS solution containing 5% (w/v) dried milk. The staining was carried out by immersing the nitrocellulose strip for 90 s in a solution of 3,3'-diaminobenzidine (0.6 mg/ml) in 0.1 M-Tris/HCl buffer, pH 7.6, containing 0.1% (v/v) H_2O_2 .

All analytical-grade reagents were purchased from Merck (Darmstadt, Germany) or Sigma Chemical Co. (St. Louis, MO, U.S.A.).

RESULTS AND DISCUSSION

Purification

Table 1 shows the procedure for the purification to homogeneity of phosphate-dependent glutaminase from a highly malignant ascitic tumour strain. The specific activity of the final preparation was apparently increased 210-fold over the original cell-free extract, and about 10-fold over the mitochondria-free extract. These values were probably underestimated, since an appreciable part of the enzyme activity was actually lost by denaturation after the enzyme extraction by the detergent from the mitochondrial inner membrane. After incubation of purified enzyme for 4 h at 4°C in the presence of 10 mM-borate/200 mM-phosphate/100 mM-pyrophosphate, pH 8, only 25% of the total glutaminase activity was found in the void volume of the Sephacryl S-300 chromatography. Under these conditions, brain and kidney glutaminases fully polymerize, yielding aggregates of M_r over 10^7 , with a notable increase in their specific activities (Curthoys *et al.*, 1976b; Haser *et al.*, 1985). The final preparation was homogeneous; a single protein band was observed by analytical non-denaturing disc electrophoresis on a 7% (w/v) polyacrylamide gel, or by isoelectric focusing in a Pharmacia Phast system. In contrast with the enzyme obtained from pig renal cortex

Table 1. Purification of phosphate-dependent glutaminase from Ehrlich ascites carcinoma

Glutaminase activity was assayed as described in the Experimental section. The results presented are for a typical purification starting from 3×10^{10} cells, extracted from 15 mice.

Fraction	Glutaminase activity ($\mu\text{mol}/\text{min}$)	Protein (mg)	Specific activity ($\mu\text{mol}/\text{min}$ per mg of protein)	Recovery (%)	Purification (fold)
Cell-free extract	28.0	8650	0.0032	100	1
Isolated mitochondria	26.4	323	0.082	94	26
Mitochondria-free extract	13.7	214	0.064	49	20
DEAE-cellulose	8.6	27.8	0.309	31	95
Sephacryl S-300	2.8	4.2	0.675	10	210

(Kvamme *et al.*, 1970), the tumour glutaminase activity could be recovered from the gel, coincident with the R_f value of the protein band stained by Coomassie Brilliant Blue. The homogeneous preparation in buffer B retained 75% of initial activity after 30 days at 4 °C.

Molecular characterization

The M_r of the phosphate-dependent glutaminase of the Ehrlich ascitic carcinoma was repetitively estimated to be 135000 by gel filtration on a Sephacryl S-300 column. This value is similar to those reported for Tris/HCl forms of pig kidney and brain (Kvamme *et al.*, 1985) or rat kidney (Curthoys *et al.*, 1976b) and brain (Haser *et al.*, 1985) glutaminases; in contrast, the M_r value of rat liver glutaminase is rather greater (Patel & McGivan, 1984; Heini *et al.*, 1987).

The purified glutaminase showed two protein-staining bands, with M_r values of 64000 and 56000 on SDS/polyacrylamide-gel electrophoresis. These findings are in agreement with the values previously reported by Olsen *et al.* (1973) for pig kidney glutaminase. In contrast,

glutaminase from pig brain has been reported to contain a single M_r -64000 polypeptide (Svenneby *et al.*, 1973). Haser *et al.* (1985) reported that glutaminase from rat brain also contains two different peptides, of M_r 65000 and 68000, that are present in the ratio approx. 4:1. More recently the same authors, using immunoblot analysis, confirmed that brain and renal mitochondria contain two glutaminase peptides that are produced *in vivo* from a common precursor (Shapiro *et al.*, 1987).

In order to compare tumour glutaminase with the kidney-type enzyme, immunoblot analysis of mitochondria from rat kidney, mouse kidney and ascites cells was carried out by using anti-(rat kidney glutaminase) antibodies, kindly supplied by Dr. N. P. Curthoys and obtained as described by Haser *et al.* (1985) (Fig. 1). The isolated mouse and rat kidney mitochondria exhibited an identical peptide pattern, with M_r values of 68000 and 65000; some weaker bands with M_r under 61000 can be observed, probably produced by a degradation of the major peptides by the action of proteinases, as previously described by Shapiro *et al.* (1987). However, in mitochondria isolated from ascites-tumour cells, the band at M_r 68000 had almost disappeared, and two immunologically reactive peptides were found, with M_r values of 65000 and 57000, that seemed to correspond to the subunits found in SDS/polyacrylamide-gel electrophoresis for the purified enzyme. These data support the idea that tumour glutaminase behaves as a kidney-type rather than a liver-type glutaminase, since the latter cannot be directly precipitated by the antibodies produced against the isolated renal enzyme (Curthoys *et al.*, 1976a).

Kinetic properties

The activity of purified tumour glutaminase was maximal at pH 9 (Fig. 2); the shape of the pH/activity

Fig. 1. Immunoblot analysis of the glutaminase contained in mitochondria isolated from rat and mouse kidney and from ascites-tumour cells

Samples of mitochondria isolated from mouse kidney (M), rat kidney (R) and ascites-tumour cells (A) were subjected to SDS/polyacrylamide-gel electrophoresis, transferred to nitrocellulose and immunostained.

Fig. 2. Effect of pH on phosphate-glutaminase

Buffers used were 0.1 M-Tris/acetic acid (pH 6.0–6.5) and 0.1 M-Tris/HCl (pH 7.0–10.0).

curve was very similar to that of the enzyme isolated from pig kidney in Tris/HCl buffer (Kvamme *et al.*, 1970) and the HeLa-cell enzyme (Williams & Manson, 1958). This result differs from the glutaminase from rat liver, whose pH optimum ranges between 7.7 and 8.5 (Patel & McGivan, 1984).

The temperature for maximal activity of purified glutaminase was 40 °C. The Arrhenius plot displayed a straight line, with an activation energy of 58 kJ/mol and a Q_{10} value of 2.2. These values are in the range of those reported for enzymically catalysed reactions (Dixon & Webb, 1979).

The enzyme is very specific for glutamine. It is not able to hydrolyse asparagine and several organic amides assayed (butylamide, acetamide, *N*-methylacetamide, *NN'*-dimethylacetamide, benzamide, formamide, *N*-methylformamide and *NN'*-dimethylformamide). In this way, tumour phosphate-dependent glutaminase seems to be a typical glutaminase, not belonging to the group of enzymes that readily catalyses the hydrolysis of both asparagine and glutamine (Wriston & Yellin, 1973).

For the purified enzyme, the activity was completely dependent on added phosphate; 100 mM-potassium pyrophosphate and 100 mM-sulphate activated by 122 % and 31 % respectively, as compared with the activation obtained at 100 mM-phosphate. Other anions at the same concentrations (arsenate, arsenite, borate, succinate, malate, citrate, lactate) failed to activate the enzyme. In the same way, up to 10 mM organic phosphates (D-glucose 6-phosphate, D-fructose 6-phosphate, D-fructose 1,6-bisphosphate, dihydroxyacetone phosphate, carbamoyl phosphate) did not activate the enzyme when assayed in the presence of 30 mM-phosphate. The tumour glutaminase was not affected by 0.1 mM- or 1.0 mM-ammonia, which are near physiological concentrations in tumour cells (Carrascosa *et al.*, 1984), in contrast with the liver enzyme (McGivan *et al.*, 1984).

The dependence of purified glutaminase activity on phosphate and glutamine are shown in Figs. 3 and 4. The concentration-dependences for both phosphate and glutamine were sigmoidal when assayed at pH 8; the values of $s_{0.5}$ for P_i and glutamine were 48 mM and 7.6 mM, when assayed at saturating concentrations of glutamine and P_i respectively. The Hill plots of these data had a slope of 1.6 for phosphate, and 1.5 for glutamine. Similar concentration-dependence curves and Hill plots were found when the enzyme was assayed at the optimum pH 9 (results not shown). Nevertheless, the $s_{0.5}$ for glutamine increased to 35 mM in the presence of non-saturating concentrations of P_i (20 mM). Shapiro *et al.* (1982) reported similar results for the glutaminase of rat kidney; the K_m for glutamine decreased from 36 to 4 mM when the phosphate concentration was increased from 5 to 100 mM. The membrane fraction isolated from frozen-and-thawed broken mitochondria showed hyperbolic kinetics (results not shown), similar to those obtained by Kovačević (1974) in intact mitochondria. The high concentrations of P_i (50–70 mM) found in the mitochondria of tumour cells (Medina *et al.*, 1988) could explain the high activity of the glutaminase *in vivo*.

One possible explanation for the different kinetics found in purified enzyme and in intact or broken mitochondria, also observed by McGivan & Bradford (1983) in liver glutaminase, could be that a conformational change occurs when the enzyme was extracted from its natural environment, the mitochondrial inner

Fig. 3. Dependence of tumour purified glutaminase activity on phosphate concentration

Insert: Hill plot of the data. The ammonia released was measured at 25 °C and pH 8 as described in the Experimental section. Glutamine concentration was 20 mM.

Fig. 4. Dependence of tumour purified glutaminase activity on glutamine concentration

Insert: Hill plot of the data. The ammonia released was measured at 25 °C and pH 8 as described in the Experimental section. Phosphate concentration was 200 mM.

membrane, since positive co-operativity for both phosphate and glutamine was only observed in the purified enzyme. Further investigation in a reconstituted liposome system is needed to confirm this hypothesis.

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