Partial purification and properties of rat liver glutaminase

Mita PATEL and John D. MCGIVAN

Department of Biochemistry, University of Bristol Medical School, Bristol BS8 1TD, U.K.

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1. The mitochondrial enzyme phosphate-dependent glutaminase was partially purified from rat liver. 2. The enzyme had M_r 290 000 as judged by chromatography on Sephacryl S-300. 3. After sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the preparation, glutaminase was tentatively identified with a peptide of M_r 73 500. 4. The concentration-dependence on glutamine was highly sigmoidal, with half-maximum velocity at 22 mM-glutamine. Half-maximum activity was obtained with 5 mM-phosphate. 5. The enzyme required ammonia as an obligatory activator, in agreement with previous reports on intact and sonicated mitochondria. 6. These findings further differentiate liver glutaminase from the phosphate-dependent glutaminase present in kidney and several other tissues.

Phosphate-dependent glutaminase (EC 3.5.1.2) accounts for 90% of the glutamine-hydrolysing activity of rat liver (Horowitz & Knox, 1968). This enzyme is mitochondrial (Guha, 1961) and appears to be associated with the inner membrane (McGivan *et al.*, 1980). Liver glutaminase has been shown not to react with antisera prepared against glutaminase isolated from kidney (Curthoys *et al.*, 1976), indicating that these two enzymes are different proteins.

The regulatory properties of liver glutaminase as measured in intact or disrupted mitochondria are complex. The hormones glucagon and vasopressin increase the effective activity of glutaminase in liver cells (Joseph & McGivan, 1978a; Joseph *et al.*, 1981b) and in mitochondria prepared from hormone-treated liver (Lacey *et al.*, 1981) or from isolated hepatocytes (Corvera & Garcia-Sainz, 1983). The effective activity of glutaminase in isolated mitochondria is also increased by hypo-osmotic swelling or by treatment with EDTA (Joseph *et al.*, 1981a). It has been suggested that these regulatory effects can be attributed primarily to an alteration in the interaction of the enzyme with the mitochondrial membrane (McGivan & Bradford, 1983a). It has further been demonstrated that glutaminase activity in intact mitochondria is completely dependent on the presence of ammonia as an obligatory activator (Verhoeven *et al.*, 1963). Work on sonicated mitochondria led to the conclusion that NH_3 rather than NH_4^+ is the activating species (McGivan & Bradford, 1983b).

In order to elucidate further the regulation of glutaminase, it is necessary to purify this enzyme

from rat liver. A considerable amount of work has been done on the purification of glutaminase from kidney, brain and other tissues (for a review see Kovačević & McGivan, 1983). However, only one attempt to purify liver glutaminase has been reported. Huang & Knox (1976) achieved a 15-fold increase in specific activity from liver by fractionation of a mitochondrial extract by $(\text{NH}_4)_2\text{SO}_4$ precipitation and Sepharose chromatography. The object of the present investigation was to improve this purification procedure and to define the kinetic and molecular properties of this enzyme as far as possible.

Experimental

Glutaminase activity was measured by assaying the production of glutamate from glutamine in a stopped assay system (McGivan & Bradford, 1983b). Glutaminase was incubated with the appropriate concentrations of substrates and activators, and the reaction was stopped after a given time by the addition of HClO_4 (final concn. 3.5%, w/v). The precipitated protein was sedimented by centrifugation at 10000g for 2 min. The acid extracts were neutralized with KOH. After sedimentation of KClO_4 (by centrifugation at 10000g for 2 min), glutamate was assayed in the extracts as described by Bernt & Bergmeyer (1965). In all experiments, the glutamate initially present at zero time was also accurately determined and subtracted from all subsequent values.

Carbamoyl-phosphate synthase (ammonia) (EC 6.3.4.16) activity was assayed by measuring the in-

corporation of $^{14}\text{CO}_2$ into citrulline in the presence of added ornithine plus ornithine transcarbamoylase (McGivan *et al.*, 1976). Ornithine transcarbamoylase was assayed by measurement of the formation of citrulline from 10mM-ornithine plus 10mM-carbamoyl phosphate (McGivan *et al.*, 1974). Glutamate dehydrogenase was assayed in the direction of glutamate formation (Schmidt, 1965).

The M_r of glutaminase was estimated by Sephacryl S-300 chromatography. The glutaminase preparation was mixed with marker proteins of defined M_r as follows: catalase (240 000), alcohol dehydrogenase (140 000), bovine serum albumin (dimer 130 000, monomer 65 000) and cytochrome *c* (13 000). Blue Dextran was also included as a marker of the void volume. The protein mixture was separated on a column (100cm $\times$ 2.5cm) of Sephacryl S-300 equilibrated in 20mM-Tris/phosphate/1mM-dithiothreitol, pH 8. Catalase was assayed by the change in A_{240} caused by decomposition of H_2O_2 (Luck, 1965). Alcohol dehydrogenase activity was assayed by monitoring the rate of decrease in A_{340} in a medium containing 100mM-KCl, 20mM-Tris/HCl, 0.2mM-NADH and 1mM-acetaldehyde at pH 7.4 at 37°C. Bovine serum albumin and cytochrome *c* were assayed by their A_{280} values.

Protein was assayed either by a biuret method in crude extracts (Gornall *et al.*, 1949) or by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Partial purification of glutaminase from rat liver

Initial attempts to repeat the purification procedure of Huang & Knox (1976) were unsuccessful. In our hands, it proved impossible to extract significant glutaminase activity from freeze-dried liver mitochondria. A different method was therefore devised, based on the findings that glutaminase can be solubilized by sonification under controlled conditions and that this enzyme has a high affinity for DEAE-cellulose.

Phosphate-dependent glutaminase was partially purified from rat liver by the following procedure (Table 1). Mitochondria were isolated from rat liver (80g wet wt.) essentially as described by Chappell & Hansford (1972) in a medium containing 250mM-mannitol, 10mM-Tris/HCl and 1mM-EGTA, pH 7.4. The homogenate was diluted to 500ml and rehomogenized before centrifugation. The mitochondria were washed only once.

To the final mitochondrial suspension was added 1 μg of the proteinase inhibitor antipain/ml plus 1mM-dithiothreitol, and the suspension was sonicated at 40W for 6 $\times$ 10s with a Dawe Soni-

Table 1. Partial purification of rat liver glutaminase

For details of the purification procedures see the text. Glutaminase activity was assayed by measuring glutamate formation over 15 min at pH 8.0 and 37°C in the presence of 50 mM-glutamine, 50 mM-Tris/phosphate and 2 mM- NH_4Cl as described in the Experimental section.

	Volume (ml)	Activity ($\mu\text{mol/min per ml}$)	Protein (mg/ml)	Total protein (mg)	Total activity ($\mu\text{mol/min}$)	Specific activity ($\mu\text{mol/min per mg}$)	Yield (%)	Purification (fold)
Homogenate	500	0.256	35	17450	128	0.0073	100	
Mitochondria	38.3	1.55	39.5	1513	59.5	0.039	46.5	5.3
Supernatant after sonication	32.6	1.13	33	1079	36.8	0.034	28.7	4.7
DEAE-cellulose fraction	7.9	1.88	4.6	36.2	14.9	0.412	11.6	56.4

probe at 4°C. The suspension was cooled in ice after each sonication period. This procedure solubilized the enzyme, but with a considerable decrease in total activity. The sonicated suspension was then centrifuged at 40000g for 30 min, and the supernatant was adjusted to pH 8.0 and KOH and applied to a column (8 cm × 2.5 cm) of DEAE-cellulose equilibrated in 20 mM-Tris/phosphate/1 mM-dithiothreitol at pH 8.0 and 4°C. The column was washed through with 50 ml of the same medium, and was then eluted with a linear gradient of 0–200 mM-KCl in 20 mM-tris/phosphate/1 mM-dithiothreitol, pH 8.0. The total volume of the gradient was 250 ml. The glutaminase peak was concentrated by dialysis against 30% (w/v) poly(ethylene glycol) in 20 mM-Tris/phosphate/1 mM-dithiothreitol, pH 8.0, at 4°C and was rapidly frozen in small fractions in liquid N₂. The enzyme preparation retained activity for up to 1 month if stored frozen, but the enzyme was unstable when thawed; 50% of the enzyme activity was lost on incubation at 37°C for 2 h or on incubation at 4°C for 12 h.

The specific activity of the final preparation was apparently increased 60-fold over the original liver homogenate (Table 1). This value is, however, probably an underestimate of the true degree of purification, since other enzymes probably contributed to the formation of glutamate from glutamine observed in homogenates. The final recovery of activity was 11%. The preparation was con-

taminated with a high activity of glutamate dehydrogenase (1.44 μmol/min per mg), but contained negligible activity of ornithine transcarbamoylase or carbamoyl phosphate synthase. It proved impossible to purify the enzyme to a higher specific activity than this. The major reasons for this were (a) the enzyme could not be satisfactorily separated from the major contaminant glutamate dehydrogenase on the basis of either charge (by DEAE-cellulose chromatography) or M_r , and (b) the preparation irreversibly lost activity on dilution below 0.04 mg of protein/ml and hence could not be further resolved by chromatographic methods.

Several procedures commonly used for the isolation of kidney-type glutaminase proved ineffective for the liver enzyme. Liver glutaminase was inhibited by the detergents Triton, Tween, cholate and cetyltrimethylammonium bromide, and lost activity in the presence of high salt concentrations, thus precluding (NH₄)₂SO₄ fractionation. Unlike the kidney enzyme, liver glutaminase does not polymerize in the presence of phosphate/borate buffers (Huang & Knox, 1976).

Molecular properties

When the glutaminase preparation was chromatographed on Sephacryl S-300, a large percentage (typically 70–90%) of the enzyme activity was lost, as a consequence of dilution. Fig. 1 shows an experiment in which the glutaminase preparation was mixed with several different marker enzymes and chromatographed on Sephacryl S-300. The peak of glutaminase activity emerged well after the void volume but before catalase. By extrapolation of the calibration curve for the column, the M_r of glutaminase was estimated to be approx. 290 000. In three such experiments values in the range 270 000–310 000 were obtained. Although these data do not allow an accurate measurement of M_r , the results are in clear contrast with the apparent M_r of 170 000 determined by sucrose-gradient centrifugation (Huang & Knox, 1976). It was not possible to use gel chromatography to separate glutaminase from glutamate dehydrogenase [M_r 350 000 (King & Frieden, 1970)].

When the enzyme preparation was separated on sodium dodecyl sulphate/polyacrylamide gels (Fig. 2), eight bands were obtained. Of these, the major band (band 6) was identical with bovine liver glutamate dehydrogenase standards. To determine which band corresponded to glutaminase subunits, the enzyme preparation was chromatographed on Sephacryl S-300 without added standards, and the glutaminase peak was identified. Various fractions from this peak were separated on gels as described in Fig. 2. After being stained, the gels were

Fig. 1. Chromatography of glutaminase on Sephacryl S-300

The glutaminase preparation was mixed with various M_r standards as described in the Experimental section, and chromatographed on Sephacryl S-300. Fractions of volume 5 ml were collected, starting after the Blue Dextran peak. The fraction number in which the peak of the various proteins emerged is plotted against the logarithm of the M_r . The glutaminase peak is indicated by the arrow.

Fig. 2. Characterization of the glutaminase preparation by gel electrophoresis

Samples of the final glutaminase preparation obtained as shown in Table 1 were dissolved in 10% sodium dodecyl sulphate and separated on sodium dodecyl sulphate/12%-polyacrylamide gels as described by Laemmli (1970). The gels were calibrated by use of suitable M_r standards. The gels were stained in 0.1% Coomassie Blue. The M_r values of the major bands identified were as follows: band 1, 90300; band 2, 83300; band 3, 73500; band 4, 66200; band 5, 63200; band 6, 53800; band 7, 49000; band 8, 42700. Band 6 ran in a position identical with that of glutamate dehydrogenase standards.

scanned, and for each fraction the areas under the peaks corresponding to bands 1–8 (Fig. 2) were compared with glutaminase activity in the same fraction.

Table 2 shows the results of such an experiment. The areas under band 3 (M_r 73500) in the various fractions corresponded closely to the glutaminase activity in those fractions. Band 6 represented glutamate dehydrogenase. The elution profile of bands 1, 2, 5, 7 and 8 was clearly different from that of glutaminase activity. The elution profile of band 4 was qualitatively somewhat similar to that

of glutaminase activity, but there was no close quantitative relationship between glutaminase activity and the area under peak 4 on the scans. On the basis of these considerations, glutaminase was tentatively identified with peak 3, and was assumed therefore to have a subunit M_r of 73500. These results, together with those in Fig. 1, are consistent with the postulate that rat liver glutaminase is composed of four subunits.

Kinetic properties

When the enzyme preparation was incubated with a near-saturating concentration of glutamine in the presence of optimal concentrations of its activators, there was a lag period of approx. 40 s at 37°C at pH 8.0 before glutamate formation was detected, after which the time course of the reaction was linear. This lag period was not greatly affected by preincubation of the enzyme with phosphate plus NH_4Cl , but was abolished when the enzyme was preincubated with glutamine (plus NH_4Cl) and the reaction started by the addition of phosphate. No satisfactory explanation for this lag can be proposed at present. It is possible that binding of glutamine converts the enzyme slowly into a more active form.

The dependence of glutaminase activity on phosphate and glutamine in the presence of saturating ammonia concentrations is shown in Fig. 3. The activity was completely dependent on added phosphate, with a half-maximum velocity at 5 mM-phosphate, in accordance with previous data (Huang & Knox, 1976). The concentration-dependence on glutamine was highly sigmoidal, with relatively little activity at 10 mM-glutamine and half-maximum velocity at 22 mM-glutamine. A Hill plot of these data (not shown) had a slope of 2.1.

It has been shown that glutaminase activity in intact mitochondria is completely dependent on added NH_4Cl (Verhoeven *et al.*, 1983), and the properties of the ammonia activation of mitochondrial glutaminase in a crude sonicated mitochondrial extract have been described (McGivan & Bradford, 1983b). Fig. 4 shows the time course of the glutaminase reaction at three different pH values measured with saturating concentrations of glutamine plus phosphate but in the absence or in the presence of 4 mM- NH_4Cl . NH_4Cl had a large activating effect at pH 7.2, a lesser effect at pH 7.5 and no apparent effect at pH 8.0. The glutamine used was found to contain approx. 0.3% ammonia as an impurity. It was concluded, in agreement with previous results, that the effectiveness of a given concentration of NH_4Cl as an activator increases with increasing pH. At pH 8.0 the ammonia present in the glutamine (approx. 0.15 mM for 50 mM-glutamine) is sufficient to

Table 2. Identification of glutaminase on sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms

An entire preparation of glutaminase as shown in Table 1 was chromatographed on a column of Sephacryl S-300 (30cm $\times$ 2.7cm) equilibrated and eluted with 20mM-Tris/phosphate/1 mM-dithiothreitol, pH8.0; 1.5ml fractions were collected. The glutaminase peak emerged between fractions 14 and 34 after the void volume, with the peak activity in fraction 22. Various fractions from this column were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as described in the legend to Fig. 2. The gels were stained with Coomassie Blue. Densitometric scans were performed, and the areas under the various bands 1–8 were obtained by integrations with a gel-scanning apparatus linked to a Hewlett–Packard 9845A computer. Bands 1–8 in the Table represent those defined in Fig. 2.

Fraction no.	Glutaminase activity (nmol/min per ml)	Peak no. ...	Area under peak (arbitrary units)							
			1	2	3	4	5	6	7	8
9	0		3.1	0.1	0.3	0.6	6.8	0	0	0
18	110		3.8	10.8	4.9	1.2	2.7	21.2	0	5.6
20	210		4.6	11.9	7.9	3.2	3.6	29.8	0	5.5
22	340		9.4	12.9	12.8	3.7	6.1	34	0	7
25	190		5.9	4.7	8.2	1.3	4	24	0	5.2
40	0		2.1	1.5	0.8	2.3	10.9	13.5	8.7	4

Fig. 3. Dependence of glutaminase activity on the concentrations of glutamine and phosphate

In (a), glutaminase (final concn. 0.15mg of protein/ml) was incubated with 50mM-phosphate, 2mM-NH₄Cl and various concentrations of glutamine at pH8.0 and 37°C. Glutamate formation was assayed after 15min. In (b) incubations were with 50mM-glutamine plus 2mM-NH₄Cl and various concentrations of phosphate.

saturate the enzyme. This finding probably accounts for the failure of previous investigators to detect the requirement of liver glutaminase for ammonia.

In further experiments (results not shown) the enzyme was titrated with NH₄Cl in the presence of saturating glutamine and phosphate, and the time course of glutamate production was followed. The concentrations of NH₄Cl required to give half-maximum activation at various pH values was estimated by computer simulation as described previously (McGivan & Bradford, 1983b). The concentrations of NH₄Cl required for half-maximum

activation were 1.2mM at pH7.0, 0.9mM at pH7.2 and 0.4mM at pH7.5. These values correspond to concentrations of free ammonia of 6.6 μ M, 7.8 μ M and 6.8 μ M respectively. These results are consistent with the value of 8 μ M-NH₃ required for half-maximum activation at a range of different pH values in crude sonicated mitochondrial extracts (McGivan & Bradford, 1983b). The present results therefore confirm the interpretation that glutaminase is activated by NH₃ rather than NH₄⁺. It follows also that the effects of NH₃ are probably exerted on the enzyme itself rather than being mediated via other components of the crude

Fig. 4. Effect of added NH_4Cl on the time course of the glutaminase reaction

Glutaminase (final concn. 0.13 mg of protein/ml) was incubated with 50 mM-glutamine plus 50 mM-Tris/phosphate in the absence (○) and in presence (●) of 4 mM- NH_4Cl . The initial endogenous ammonia concentration was 0.2 mM. (a) pH 8.0; (b) pH 7.5; (c) pH 7.2.

sonicated mitochondrial extracts studied previously.

The maximum activity of the enzyme was virtually constant between pH 7.7 and 8.5, but decreased strongly below pH 7.6 (see also Huang & Knox, 1976). The temperature-dependence, measured in the presence of maximum concentrations of activators under conditions that eliminated possible errors caused by the initial non-linearity of the time courses, gave a linear Arrhenius plot with an activation energy of 67 kJ/mol. This is comparable with the situation in sonicated mitochondrial extracts, but distinct from that in frozen-and-thawed mitochondria, where the Arrhenius plot was discontinuous (McGivan *et al.*, 1980).

The rate of glutamine hydrolysis in both intact mitochondria and mitochondria disrupted by freezing and thawing is stimulated by HCO_3^- (Joseph & McGivan, 1978b; McGivan *et al.*, 1980), and this activation had been assumed to be mediated by a direct effect on glutaminase. With the partially purified enzyme preparation, it was found that at pH 7.4 the addition of 25 mM-bicarbonate in the absence of added ammonia increased the activity of glutaminase to that obtained when saturating ammonia was present

(results not shown). There was no effect of added bicarbonate at pH 8 in either the presence or the absence of added ammonia. These results suggest that bicarbonate does not activate glutaminase directly. The apparent activating effect seen in the absence of added ammonia at pH 7.4 can probably be ascribed to a primary effect of bicarbonate in increasing the pH of the medium (due to loss of CO_2) and hence potentiating the effect of the sub-saturating endogenous ammonia in the glutamine. Leucine has also been shown to stimulate glutamine hydrolysis in intact hepatocytes (Baverel & Lund, 1979). However, leucine at concentrations up to 5 mM failed to activate the partially purified enzyme, and hence the explanation for the activating effect of this compound on glutamine hydrolysis in intact hepatocytes must lie elsewhere.

Discussion

The present paper describes a method for the partial purification of rat liver glutaminase. The purification achieved here is 4 times that achieved previously, but no satisfactory method for removing glutamate dehydrogenase, the major contaminant, could be found. The success of the purification procedure was also limited by the fact

that the enzyme is unstable after detachment from the mitochondrial membrane, and is very sensitive to inhibition by dilution. It is probable that the enzyme purified here is identical with that studied previously by Huang & Knox (1976), although the glutaminase activity reported by those authors in liver homogenates and mitochondrial fractions is at least 5 times that measured in the present work. The M_r determined by Huang & Knox (1976) was 170000, compared with the value of 290000 determined here.

The differences between liver-type and kidney-type phosphate-dependent glutaminases first noted by Krebs (1935) and later studied by Horowitz & Knox (1968), Huang & Knox (1976) and Curthoys *et al.* (1976) are further emphasized here. Liver glutaminase has a much lower phosphate requirement than has the kidney enzyme. The kidney enzyme shows hyperbolic kinetics with respect to glutamine concentration, with a K_m of 4–5 mM (Klingman & Handler, 1958), whereas the liver enzyme has highly sigmoidal kinetics, with half-maximum velocity at 22 mM-glutamine. The particular distinguishing feature of liver glutaminase is its requirement for ammonia as an obligatory activator, which has now been demonstrated in intact mitochondria (Verhoeven *et al.*, 1983), sonicated mitochondria (McGivan & Bradford, 1983a) and with the partially purified enzyme. Liver glutaminase also has a high net negative charge at pH 8, as shown by its affinity for DEAE-cellulose. The time course of glutamate production shown in Fig. 3 amply confirms the original finding by Krebs (1935) that liver glutaminase is not inhibited by glutamate.

The molecular properties of the liver and kidney enzymes are also different. Kidney glutaminase in the absence of phosphate has M_r 160000, but it dimerizes in the presence of phosphate, and polymerizes to a form of $M_r > 2 \times 10^6$ in phosphate/borate buffer (Godfrey *et al.*, 1977). The liver enzyme appears to be a tetramer with an apparent M_r of 290000 as judged by Sephacryl chromatography and a presumed subunit M_r of 73500. This enzyme does not polymerize (Huang & Knox, 1976).

A major purpose of the present study was to obtain a preparation of purified glutaminase that could be used to study the interaction of this enzyme with the liver mitochondrial inner membrane. In frozen-and-thawed mitochondria, where the enzyme is associated with the inner membrane, the glutamine-concentration-dependence is hyperbolic, with a K_m of 6 mM (McGivan & Bradford, 1983a). The enzyme also exhibits 70% of its maximum activity in the absence of phosphate. On detachment of the enzyme from the membrane by phospholipase treatment, the kinetic properties

revert to those of the isolated enzyme described in the present paper. The detailed kinetic properties of this enzyme in frozen-and-thawed mitochondria and possibly in intact mitochondria are therefore clearly a function of the binding of the enzyme to the mitochondrial membrane.

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