Factors influencing the inactivation of phosphate-dependent glutaminase in the matrix fraction of rat liver mitochondria

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1. The activity of phosphate-dependent glutaminase was measured in a matrix extract of essentially lysosome-free liver mitochondria. 2. ATP, GTP or a non-hydrolysable analogue of ATP stimulated the rapid inactivation of glutaminase, but not of other matrix enzymes. 3. Glutaminase was protected against inactivation if high concentrations of glutamine or low concentrations of NH_3 were present. 4. Inactivation of glutaminase in the presence of ATP did not markedly affect the reaction of the enzyme with a specific polyclonal antiserum. 5. These results in a mitochondrial extract are similar to the characteristics of glutaminase inactivation in intact hepatocytes, suggesting a similar mechanism in each case. 6. The presence of a specific ATP-activated protease in the mitochondrial matrix is suggested to be responsible for glutaminase inactivation.

INTRODUCTION

The important regulatory enzyme phosphate-dependent glutaminase (EC 3.5.1.2) is located in the liver mitochondrial matrix. The enzyme is induced by glucagon in isolated hepatocytes, but only if either NH_3 or high concentrations of glutamine are present in the culture medium (see the preceding paper; McGivan *et al.*, 1991). Glutaminase activity decreases rapidly on culturing hepatocytes for 24–40 h, or *in vivo* on transplanting ascites tumours into mice (Quesada *et al.*, 1988).

Factors determining the breakdown of mitochondrial-matrix enzymes are relatively poorly understood. Although the degradation of mitochondria in general is considered to occur in the lysosomes (Glaumann *et al.*, 1981), it is likely that individual mitochondrial enzymes with a rapid turnover are also degraded extralysosomally. A number of different extralysosomal proteases occur in mammalian cells (for a review see Bond & Butler, 1987). In particular, cytosolic proteases have been identified in rat liver (DeMartino & Goldberg, 1979; Rivett, 1985; Tanaka *et al.*, 1986).

Mitochondrial-matrix proteases have been implicated in the turnover of the liver mitochondrial enzymes carbamoyl phosphate synthase (Nicoletti *et al.*, 1977) and ATPase (Perez-Pastor *et al.*, 1982). An ATP-dependent vanadate-sensitive endoprotease has been identified in the matrix of rat liver mitochondria (Desautels & Goldberg, 1982). Since liver phosphate-dependent glutaminase is rapidly inactivated under certain conditions both in isolated hepatocytes and *in vivo*, it was of interest to determine whether this inactivation might be catalysed by a similar protease located in the mitochondrial matrix.

In the present investigation, the inactivation of phosphate-dependent glutaminase in the matrix fraction of lysosome-free liver mitochondria is characterized.

MATERIALS AND METHODS

Materials

Biochemicals and digitonin were purchased from Sigma. Enzymes were from Boehringer. Materials for Western blotting were from Bio-Rad. All other chemicals were purchased from BDH and were of AnalaR grade.

Preparation of a lysosome-free matrix extract of mitochondria

Mitochondria were prepared from the livers of normally fed 300 g male Wistar rats in a medium containing 0.25 M-sucrose, 10 mM-Tris/HCl and 1 mM-EGTA at pH 7.4. After sedimentation at 10000 *g* for 10 min, the mitochondrial pellet was rehomogenized, washed twice by sedimentation at 10000 *g* for 10 min and finally resuspended at a protein concentration of 20 mg of protein/ml in the same medium at 4 °C. An equal volume of medium containing 4 mg of digitonin/ml was added to disrupt lysosomes without affecting the integrity of the mitochondrial inner membrane. After stirring for 2 min, the suspension was sedimented at 10000 *g* for 10 min and the pellet washed twice. The mitochondria were then sonicated at 40 W for 6 × 20 s, and the sonicated material was centrifuged at 100000 *g* for 30 min at 4 °C. The pellet was discarded. The purified matrix fraction was assayed for protein and glutaminase activity and frozen in small batches in liquid N_2 . The activity was stable for at least 3 weeks when frozen. Glutaminase activity in this extract was in the range 180–300 nmol/min per mg of protein at 37 °C when measured under optimal conditions.

For the measurement of glutaminase inactivation, mitochondrial extract was routinely incubated at 37 °C in a medium containing 30 mM-potassium phosphate, pH 7.5, plus 3 mM-EDTA, together with further additions as shown. At appropriate intervals, samples were withdrawn and assayed for enzyme activity. Glutaminase activity was assayed over periods of 10–30 min in a medium containing 170 mM-glutamine, 100 mM-potassium phosphate, pH 8.0, 1.5 mM- NH_4Cl and 0.15–0.25 mg of extract (Heini *et al.*, 1987). This concentration of NH_4Cl is saturating for the activation of glutaminase at this pH. The reaction was terminated by addition of trichloroacetic acid, and the NH_3 produced was assayed by using the *o*-phthalaldehyde/mercaptoethanol reagent (see Heini *et al.*, 1987).

For each measurement, a zero-time value was obtained by incubating the incubation medium containing the mitochondrial extract with the assay medium and adding the trichloroacetic acid immediately. Control experiments showed that when NH_4Cl was added at concentrations between 0.2 and 10 mM to the incubation medium containing the extract, the rate of glutaminase activity was unaffected when calculated as the NH_3 formed in a given time minus that in the extract at zero time.

Malate dehydrogenase (Bergmeyer & Bernt, 1965) and glutamate dehydrogenase (Schmidt, 1965) were measured by standard spectrophotometric methods. Ornithine aminotransferase activity was assayed as described by Peraino & Pitot (1964). Acid phosphatase activity was assayed as described by Saini & van Etten (1979). ATP was assayed spectrophotometrically by using glucose and hexokinase (Lamprecht & Trautschold, 1965).

Western blotting was performed as described by Towbin *et al.* (1979). The mitochondrial extract was incubated with 30 mM-phosphate plus 3 mM-EDTA at pH 7.5, with or without 10 mM-ATP for 2.5 h. After this time, 20 µg of protein was separated by SDS/PAGE, and the proteins were transferred to nitrocellulose. After blocking, rabbit anti-(rat liver glutaminase) antiserum was added. After incubation and washing, the antibody was located with peroxidase-labelled goat anti-rabbit IgG. For immunoprecipitation experiments, the mitochondrial extracts which had been preincubated with or without ATP as above were incubated with appropriate amounts of the antibody at 4 °C for 1 h. Then 0.1 ml of a suspension of crude Protein A (*Staphylococcus aureus*; Sigma Chemical Co.) was added and after a further 15 min the Protein A-antibody-glutaminase complex was sedimented at 10000 *g* for 5 min. Glutaminase activity was measured in the supernatant. Control experiments were performed without antibody and without Protein A.

RESULTS

Characteristics of ATP-stimulated decrease in glutaminase activity

In order to assess the lysosomal contamination of the extract used, the lysosome-specific enzyme acid phosphatase was assayed in various fractions during the preparation (Table 1). Mitochondria prepared by conventional differential centrifugation contained a specific activity of acid phosphatase similar to that in the original homogenate. This was greatly decreased on lysing the lysosomes with digitonin and washing. After further sonication and centrifugation at high speed, the final extract contained only a very low activity of this enzyme.

In lysosome-free mitochondrial-matrix extracts, glutaminase activity exhibited a slow time-dependent decrease when incubated over a period of several hours at 37 °C. This decrease in activity was abolished by the addition of GSH (Fig. 1) or other thiol reagents such as cysteine or mercaptoethanol (results not shown), and was presumably due to the slow oxidation of an essential thiol group in the enzyme. When ATP was added in the presence of EDTA to prevent ATP hydrolysis, glutaminase activity

Fig. 1. Inactivation of glutaminase in the presence or absence of ATP and GSH

The mitochondrial extract was incubated with 30 mM-potassium phosphate, pH 7.5, and 3 mM-EDTA. The final protein concentration was 8 mg/ml. Further additions were: ■, none; ▲, 15 mM-GSH; △, 10 mM-ATP (Na⁺ salt); ●, 15 mM-GSH plus 10 mM-ATP. Glutaminase activity was assayed in samples taken after the times shown. The values shown are means $\pm$ S.E.M. of triplicate incubations.

underwent a progressive and rapid decrease with time, to 30 % of the initial value in 2 h (Fig. 1). This decrease in activity brought about by ATP was neither prevented nor reversed by the presence of GSH, indicating that the effect was not a trivial one owing to increased exposure and oxidation of thiol groups. The effect was not due to a simple direct inhibition of glutaminase activity by ATP since no effect was observed on the immediate addition of ATP, and the decrease in glutaminase activity was progressive over a prolonged period.

When 10 mM-ATP was added to the mitochondrial extract in the absence of EDTA, the ATP was rapidly hydrolysed, owing to endogenous Mg²⁺-stimulated ATPase activity in the extract. In a typical experiment, the ATP concentration in the medium fell from 10 mM to less than 0.1 mM in the first 30 min. Glutaminase activity was initially 207 nmol/min per mg, but decreased to 160 nmol/min per mg after 30 min, and this activity remained virtually constant over the next 2 h. In the presence of 3 mM-EDTA the ATP concentration decreased from 10 mM to 9.5 mM over 2.5 h, and the glutaminase activity decreased from 207 to

Table 1. Acid phosphatase activity in mitochondrial extracts

Acid phosphatase activity was measured during a 2 min incubation in a buffer containing 100 mM-NaCl and 50 mM-sodium acetate at 37 °C and pH 5.0 with 5 mM-*p*-nitrophenol as substrate. Assays were performed on the initial liver homogenate, the initial mitochondrial fraction, mitochondria incubated with digitonin and the final extract. The values shown are the means $\pm$ S.E.M. for preparations from three separate livers.

Fraction	Acid phosphatase activity (nmol/min per mg of protein)
Homogenate	61.9 $\pm$ 1.8
Mitochondria	54.1 $\pm$ 1.7
Mitochondria, digitonin-treated and washed	14.4 $\pm$ 2.0
Final matrix extract	1.5 $\pm$ 0.8

Fig. 2. Effect of ATP[S] on glutaminase inactivation

The experiment was performed as in the legend to Fig. 1. Additions were: ▲, none; ■, 5 mM-ATP[S]; ●, 10 mM-ATP[S]; △, 10 mM-ATP. The values shown are means $\pm$ S.E.M. of triplicate incubations.

Table 2. Nucleotide specificity for glutaminase inactivation

The mitochondrial extract was incubated at 37 °C in a medium containing 30 mM-potassium phosphate, pH 7.5, 3 mM-EDTA and 5 mM of the appropriate nucleotide for 120 min. The protein concentration was 4.6 mg/ml and the initial glutaminase activity was 190 nmol/min per mg. The results represent means $\pm$ S.E.M. of triplicate incubations: * $P < 0.001$ versus incubation with no additions.

Additions (5 mM)	Activity after 2 h (nmol/min per mg)
None	162 $\pm$ 3
ATP	96.5 $\pm$ 3*
GTP	112 $\pm$ 7*
UTP	158 $\pm$ 15
ADP	162 $\pm$ 13
GDP	175 $\pm$ 12
AMP	157 $\pm$ 14
IMP	169 $\pm$ 17
Cyclic AMP	152 $\pm$ 8

61 nmol/min per mg in this time. It therefore appeared that the presence of ATP itself, rather than ATP hydrolysis, was required for glutaminase degradation. This was confirmed by incubation of the extract with the non-hydrolysable ATP analogue adenosine 5'-[γ -thio]triphosphate (ATP[S]) (Fig. 2). Increasing concentrations of ATP[S] caused a progressively faster decrease in glutaminase activity; the effect of 10 mM-ATP[S] was nearly equivalent to that of 10 mM-ATP in the presence of EDTA.

These results suggested that the presence of ATP caused a progressive inactivation of glutaminase activity, possibly via an ATP-stimulated protease activity. The characteristics of this putative protease activity were further investigated. Table 2 shows that the effect of ATP was mimicked by GTP, but CTP, UTP, dinucleotides and mononucleotides were without effect. The ATP-stimulated decrease in glutaminase activity was not prevented by the protease inhibitors antipain, pepstatin, leupeptin, benzamidine or vanadate (results not shown). Thiol-specific reagents such as *N*-ethylmaleimide, *p*-hydroxymercuribenzoate or iodoacetamide, which inhibit certain classes of proteases, could not be tested, since these compounds themselves totally inhibited glutaminase activity.

The ATP-stimulated decrease in glutaminase activity was relatively independent of pH in the pH range 7.0–7.8. Thus in a series of experiments where the extract was incubated with

Table 3. Enzyme specificity of ATP effect

A mitochondrial-matrix extract was incubated with 10 mM-ATP plus 30 mM-potassium phosphate, pH 7.5, plus 3 mM-EDTA for 180 min. Samples of the extract were assayed at zero time and at 180 min for the enzymes shown as described in the Materials and methods section. Activities are given in units of μ mol/min per mg of protein at 37 °C, and are means $\pm$ S.E.M. of triplicate incubations.

Enzyme	Activity (μ mol/min per mg)		
	Initial	After 180 min	
		No ATP	+ ATP
Glutaminase	0.205 $\pm$ 0.004	0.185 $\pm$ 0.009	0.072 $\pm$ 0.004
Ornithine aminotransferase	0.064 $\pm$ 0.004	0.060 $\pm$ 0.004	0.068 $\pm$ 0.005
Glutamate dehydrogenase	3.68 $\pm$ 0.14	3.46 $\pm$ 0.40	3.33 $\pm$ 0.20
Malate dehydrogenase	6.88 $\pm$ 0.17	6.45 $\pm$ 0.30	6.57 $\pm$ 0.60

10 mM-ATP plus 3 mM-EDTA for 100 min and subsequently assayed at pH 8, 33.3 % of the activity was lost during incubation at pH 8.0, 39.8 % at pH 7.4, and 41.2 % at pH 7.0. The loss of activity in the absence of ATP ranged from 8 % at pH 8 to 14 % at pH 7. Glutaminase is unstable and irreversibly loses activity below pH 6.5; therefore incubation at lower pH values could not be done.

The activity of a number of other matrix enzymes was monitored in the mitochondrial extract on addition of ATP (Table 3). Under conditions where glutaminase activity was decreased, there was no progressive effect of ATP on the activities of glutamate dehydrogenase, malate dehydrogenase or ornithine aminotransferase, indicating that the effect of ATP in stimulating inactivation of glutaminase was relatively specific.

Protection of glutaminase activity

In intact hepatocytes, glutaminase activity is protected by high concentrations of glutamine or by the presence of NH_4^+ ions. If the ATP-dependent loss of glutaminase activity in mitochondrial-matrix fractions has any physiological significance, a similar protection by these metabolites might be expected. Table 4 shows the effect of incubating the mitochondrial extract with various compounds in the presence of ATP plus EDTA for 160 min at 37 °C. In the absence of other additions, a large decrease in glutaminase activity was observed in the presence, but not the absence, of ATP. L-Glutamine (5 mM) partially protected the enzyme activity. Other related amino acids were without effect. Similarly 2 mM- NH_4Cl partially protected glutaminase activity.

Fig. 3 shows the concentration-dependence of the protective effect of glutamine. In the absence of phosphate, glutamine did not protect enzyme activity. The effect of 10 mM-glutamine was dependent on the concentration of phosphate; phosphate itself had only a small protective effect. In the presence of an optimum concentration of phosphate, the protective effect of glutamine was highly concentration-dependent. High glutamine concentrations were required to obtain a maximum effect.

The protection by NH_3 was further characterized. Fig. 4 shows that protection by NH_4Cl was half-maximal at less than 1 mM. The possibility that NH_3 exerted its effect by inactivating any residual lysosomes in the preparation was excluded by the fact

Table 4. Specificity of protection of glutaminase

The mitochondrial extract was incubated in the presence of 30 mM-potassium phosphate, pH 7.4, 3 mM-EDTA and other additions as shown for 160 min at 37 °C. Glutaminase activity was assayed over a 20 min time interval as described in the Materials and methods section. The initial activity was 190 nmol/min per mg. The values given are means $\pm$ S.E.M. of triplicate incubations: * $P < 0.05$ versus incubation in the presence of ATP alone. The presence of NH_4Cl in the incubation did not affect the glutaminase assay (see the Materials and methods section).

Additions	Glutaminase activity (nmol/min per mg)
No ATP	150 $\pm$ 10
10 mM-ATP	47 $\pm$ 4
ATP + 5 mM-L-glutamine	89 $\pm$ 6*
ATP + 5 mM-D-glutamine	53 $\pm$ 3
ATP + 5 mM-L-glutamate	59 $\pm$ 6
ATP + 5 mM-L-aspartate	54 $\pm$ 3
ATP + 5 mM-L-leucine	44 $\pm$ 9
ATP + 5 mM-L-alanine	33 $\pm$ 5
ATP + 5 mM-carbamoylglutamate	35 $\pm$ 7
ATP + 5 mM-N-acetylglutamate	40 $\pm$ 7
ATP + 2 mM- NH_4Cl	105 $\pm$ 8*

Fig. 3. Protection by phosphate and glutamine in the presence of ATP

The mitochondrial extract was incubated with 10 mM-ATP, 20 mM-Tris/HCl, pH 7.5, 3 mM-EDTA and concentrations of phosphate and glutamine as indicated. In (a) the concentration of phosphate (pH 7.5) was varied in the absence ($\blacktriangle$) or the presence ($\blacksquare$) of 10 mM-glutamine. In (b), 30 mM-phosphate was present throughout. Incubations were performed for 150 min, and the residual activity was assayed after correction for NH_3 produced in the incubation medium. The initial glutaminase activity in the extract was 223 nmol/min per mg. The values shown are means $\pm$ S.E.M. of triplicate incubations.

that methylamine, which has similar effects to NH_3 on lysosomes (see Seglen, 1983), had very little protective effect on glutaminase activity.

Immunological properties of the active and inactive enzyme proteins in mitochondrial extracts were compared. Fig. 5 shows a Western blot of glutaminase using a specific glutaminase antibody in mitochondrial extracts which had been incubated with or without ATP for 2.5 h. Although the extract incubated without ATP appeared to contain slightly more immunoreactive protein (M_r 59000), the intensity of the bands did not correspond quantitatively to the relative enzyme activities. No immuno-

Fig. 4. Protection of glutaminase by NH_3

The experiment was performed as described in Fig. 3. The incubation medium contained 30 mM-phosphate, 3 mM-EDTA, 10 mM-ATP and various concentrations of NH_3 ($\blacktriangle$) or methylamine ($\blacksquare$) as shown. The initial activity in this experiment was 205 nmol/min per mg. The values shown are means $\pm$ S.E.M. of triplicate incubations.

Fig. 5. Immunoblot of glutaminase in mitochondrial extracts incubated with or without ATP

The experiment was performed as described in the Materials and methods section. Equal amounts of mitochondrial protein were used for each track. Tracks: (a) protein standards; (b) and (c) mitochondria incubated without ATP; (d) and (e) mitochondria incubated with ATP. The incubation time was 150 min. The initial activity was 250 nmol/min per mg. The final activity in the samples incubated without ATP was 217 nmol/min per mg and in those incubated with ATP was 72 nmol/min per mg.

reactive peptides at lower M_r values, which might correspond to major breakdown products, were observed.

Fig. 6 shows an immunotitration experiment on the same system. Mitochondrial extract was incubated with or without ATP for 2.5 h so that the activity was decreased to 25% of its initial value. When glutaminase activity in extracts containing equal concentrations of protein was immunoprecipitated with the antibody, the antibody titrations were similar (Fig. 6a). If the titration was carried out in extracts containing equal activities of glutaminase, and therefore different protein concentrations, relatively more antibody was required to precipitate the ATP-treated sample, which contained more protein (Fig. 6b). These results indicate that the polyclonal antibody did not discriminate to an appreciable extent between active and inactivated glutaminase, and that the inactivation caused by addition of ATP does not arise from a major change in the protein structure which could be detected by differential antibody binding.

DISCUSSION

The above results establish that a factor exists in the matrix fraction of rat liver mitochondria which catalyses the inactivation of phosphate-dependent glutaminase in the presence of ATP, GTP or non-hydrolysable analogues of ATP. Glutaminase activity is protected by concentrations of NH_4Cl in the range 0–2 mM or by high concentrations of glutamine in the presence of phosphate.

A number of factors suggest that these findings in mitochondrial-matrix fractions are consistent with the regulation of glutaminase activity in cultured hepatocytes. Glutaminase activity in hepatocytes decreases rapidly over a period of hours in the presence of low glutamine concentrations. High glutamine concentrations postpone the decrease in glutaminase activity, and the continuous presence of NH_4^+ ions at concentrations above 0.4 mM protects glutaminase activity in hepatocytes. Mitochondria in intact hepatocytes contain sufficiently high concentrations of ATP to bring about rapid glutaminase ac-

Fig. 6. Immunoprecipitation of glutaminase after incubation of extract with and without ATP

The experiment was performed as described in the Materials and methods section. The initial activity was 230 nmol/min per mg. After incubation for 150 min at 37 °C, the samples incubated without ATP had an activity of 215 nmol/min per mg, whereas those incubated with ATP had an activity of 52 nmol/min per mg. In (a) the protein concentration of each sample was 6.6 mg/ml after addition of the antibody solution. In (b) the protein concentration of the sample incubated without ATP was 3.3 mg/ml, and that of the sample incubated with ATP was 13.6 mg/ml. The protein concentration of the glutaminase antiserum was 3.3 mg/ml. ■, Samples incubated with ATP; ▲, samples incubated without ATP. The values shown are means $\pm$ S.E.M. of triplicate incubations.

tivation if NH_3 is absent. It is unclear whether the degradation of glutaminase occurs simply in response to lack of protection of NH_3 , or whether the degradative process had to be triggered by some other factor when NH_3 levels are low.

The mechanism by which glutaminase is inactivated in mitochondrial-matrix fractions cannot be positively identified from the present results. Since ATP does not itself inhibit the enzyme, it follows that ATP must stimulate some other glutaminase-inactivating enzyme activity. One possibility is the presence in the matrix of a previously undescribed ATP-stimulated protease which attacks glutaminase, but not glutamate dehydrogenase, ornithine aminotransferase or malate dehydrogenase. If such a protease exists, it is different from the ATP-dependent mitochondrial-matrix protease identified by Desautels & Goldberg (1982), the action of which requires ATP hydrolysis. This previously described protease was inhibited by vanadate and was not stimulated by non-hydrolysable analogues of ATP. ATP-stimulated cysteine proteases which do not involve ATP hydrolysis and which are vanadate-independent and activated by ATP analogues have been identified in liver cytosol (DeMartino & Goldberg, 1979; Rose *et al.*, 1979), but not previously in the mitochondrial matrix. This type of enzyme is usually characterized by its sensitivity to various thiol reagents. Unfortunately, the sensitivity to these reagents of the putative protease causing inactivation of glutaminase could not be assessed, since glutaminase itself is strongly inhibited by such reagents.

Although such a mitochondrial-matrix protease could be inhibited by glutamine or by NH_4Cl , it is more likely that the presence of the substrate and obligatory activators of glutaminase (phosphate and NH_3) cause the enzyme to adopt a conformation which is not readily attacked by proteases. The modification of the enzyme which results in loss of activity is not detected by the polyclonal antibody used, which suggests that, if proteolytic degradation of the enzyme occurs, the initial stage does not cause major changes in enzyme structure, although activity is lost.

These findings provide a rationale for the rapid loss of activity of glutaminase in intact cells and in the liver *in vivo* and its protection by NH_3 and high concentrations of glutamine in hepatocytes. In view of the fact that the antigenic determinants of the protein are lost during activity loss in hepatocytes (see the accompanying paper; McGivan *et al.*, 1991), although the rapid inactivation in mitochondrial extracts does not involve much change in immunological properties of the enzyme, it may be speculated that the initial change in the enzyme which occurs in mitochondria may make the enzyme protein more susceptible to subsequent export from the mitochondria and to further extensive degradation in the lysosomes.

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