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

John D. McGIVAN, Frances A. DOYLE and Kevin BOON
Department of Biochemistry, School of Medical Sciences, University Walk, Bristol BS8 1TD, U.K.

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 NH₃ 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 24–48 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 AnalR 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 N₂. 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-NH₄Cl and 0.15–0.25 mg of extract (Heini et al., 1987). This concentration of NH₄Cl is saturating for the activation of glutaminase at this pH. The reaction was terminated by addition of trichloroacetic acid, and the NH₃ produced was assayed by using the o-phthalaldialdehyde/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 NH₄Cl 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 NH₃ 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 Sainsi & 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 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

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Acid phosphatase activity (nmol/min per mg of protein)</th>
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<tbody>
<tr>
<td>Homogenate</td>
<td>61.9 ± 1.8</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>54.1 ± 1.7</td>
</tr>
<tr>
<td>Mitochondria, digitonin-</td>
<td>14.4 ± 2.0</td>
</tr>
<tr>
<td>treated and washed</td>
<td></td>
</tr>
<tr>
<td>Final matrix extract</td>
<td>1.5 ± 0.8</td>
</tr>
</tbody>
</table>

![Fig. 1. Inactivation of glutaminase in the presence or absence of ATP and GSH](image-url)

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 ± S.E.M. of triplicate incubations.

![Fig. 2. Effect of ATP[S] on glutaminase inactivation](image-url)

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 ± 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 ± S.E.M. of triplicate incubations: *P < 0.001 versus incubation with no additions.

<table>
<thead>
<tr>
<th>Additions (5 mM)</th>
<th>Activity after 2 h (nmol/min per mg)</th>
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<tbody>
<tr>
<td>None</td>
<td>162 ± 3</td>
</tr>
<tr>
<td>ATP</td>
<td>96.5 ± 3*</td>
</tr>
<tr>
<td>GTP</td>
<td>112 ± 7*</td>
</tr>
<tr>
<td>UTP</td>
<td>158 ± 15</td>
</tr>
<tr>
<td>ADP</td>
<td>162 ± 13</td>
</tr>
<tr>
<td>GDP</td>
<td>175 ± 12</td>
</tr>
<tr>
<td>AMP</td>
<td>157 ± 14</td>
</tr>
<tr>
<td>IMP</td>
<td>169 ± 17</td>
</tr>
<tr>
<td>Cyclic AMP</td>
<td>152 ± 8</td>
</tr>
</tbody>
</table>

61 nmol/min per mg in this range. 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 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.

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 mmol/min per mg of protein at 37 °C, and are means ± S.E.M. of triplicate incubations.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity (μmol/min per mg)</th>
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<tbody>
<tr>
<td></td>
<td>Initial</td>
</tr>
<tr>
<td>Glutaminase</td>
<td>0.205 ± 0.004</td>
</tr>
<tr>
<td>Ornithine aminotransferase</td>
<td>0.064 ± 0.004</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>3.68 ± 0.14</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>6.86 ± 0.17</td>
</tr>
</tbody>
</table>

Protection of glutaminase activity

In intact hepatocytes, glutaminase activity is protected by high concentrations of glutamine or by the presence of NH₄⁺ 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-NH₄Cl 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 NH₄Cl was further characterized. Fig. 4 shows that protection by NH₄Cl was half-maximal at less than 1 mM. The possibility that NH₄Cl exerted its effect by inactivating any residual lysosomes in the preparation was excluded by the fact that protection by NH₄Cl was partial at 1 mM.
that methylamine, which has similar effects to NH₄ 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 (M₀, 59000), the intensity of the bands did not correspond quantitatively to the relative enzyme activities. No immuno-

**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 NH₄Cl 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 NH₄⁺ 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-
Mitochondrial inactivation of glutaminase

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 32 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 ± S.E.M. of triplicate incubations.

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 NH₃ and high concentrations of glutamine in hepatocytes. In view of the fact that the antigens 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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REFERENCES


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