Localization and some properties of phosphate-dependent glutaminase in disrupted liver mitochondria

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1. Glutaminase activity in frozen and thawed liver mitochondria was activated by NH₄⁺, phosphate and HCO₃⁻ ions and also by ATP. 2. NH₄⁺ and HCO₃⁻ ions decreased the requirement of the enzyme for phosphate. The activation by ATP was observed only in the presence of NH₄⁺ or HCO₃⁻ ions. 3. In frozen-and-thawed mitochondria, the enzyme was loosely bound to the inner membrane, the Arrhenius plot showing a break at 23°C. On sonication, glutaminase was detached from the membrane and the Arrhenius plot became linear. 4. The apparent Kₘ for glutamine of the membrane-bound form was 6 mM, and that of the soluble form was 21 mM. 5. It is likely that the properties of glutaminase in the intact cell are dependent on the association of this enzyme with the mitochondrial membrane.

The properties of phosphate-dependent glutaminase (EC 3.5.1.2) isolated from kidney have been extensively investigated (e.g. Kvamme et al., 1970; Svenneby et al., 1970), as have the intracellular location of this enzyme (Curthoys & Weiss, 1974) and the characteristics of glutaminase activity in intact and disrupted kidney mitochondria (e.g. Kovacevic, 1976; Kovacevic et al., 1979). In comparison, the liver enzyme has been little studied. Kalra & Brosnan (1973) showed that glutaminase could be released from liver mitochondria in a soluble form by sonication or detergent treatment. Some properties of the isolated enzyme have been reported (Huang & Knox, 1976).

Evidence has been presented that addition of glucagon to liver cells activates intramitochondrial glutaminase (Joseph & McGivan, 1978a), and that glutamine is a substrate for gluconeogenesis at low concentrations only when glucagon is present. Some properties of glutaminase activity in intact liver mitochondria have been described (Joseph & McGivan, 1978b), and it is clear that the regulation of this enzyme is complex.

The eventual aim of the present investigation is to clarify the mechanism by which glucagon activates glutaminase in liver cells. As a first step, this paper reports the properties of glutaminase in disrupted mitochondria.

Materials and methods

Preparation and disruption of mitochondria

Mitochondria were prepared from the livers of 200g female Wistar rats by the method of Chappell & Hansford (1972) in a medium containing 0.25M-mannitol, 5 mM-Tris/HCl and 1 mM-EGTA at pH 7.4. Disruption of the mitochondria was achieved by two different methods: after addition of an equal volume of water, the mitochondrial suspension was frozen and thawed three times in an acetic solid-CO₂ bath, or sonicated for 6 × 15s at 100W with a Soniprobe (Dawe Instruments) at 4°C.

Measurement of glutaminase activity

Glutaminase activity was routinely measured by assaying the formation of glutamate from glutamine. In disrupted mitochondria, glutamate is not further metabolized (Joseph & McGivan, 1978b). Unless otherwise stated, the disrupted mitochondria were incubated at 30°C and pH 7.4 for 10min in a medium containing Tris/HCl (10 mM final concn.) and concentrations as indicated of glutamine, NH₄Cl, KHCO₃, potassium phosphate, or ATP plus oligomycin. The formation of glutamate was found to be linear with time over this period. Incubations were terminated by the addition of HCl (3.5%, w/v, final concn.). After centrifugation at 10000g for 2 min, the supernatant was neutralized with 3M-KOH and assayed for glutamate by the method of Bernt & Bergmeyer (1965).

Measurement of enzyme distribution

Mitochondria were disrupted as described above, and the suspension was centrifuged at 40000g for 30min. The pellet was resuspended in mitochon-
dria-preparation medium, and the volumes of the supernatant and pellet fractions were noted. In these fractions, and in a sample of the original suspension, the following enzymes were assayed at pH 7.4: citrate synthase (Sørensen et al., 1963), glutamate dehydrogenase (Schmidt, 1965) in the presence of 2mM-ADP, and succinate–cytochrome c reductase (Tisdale, 1967). Malate dehydrogenase was assayed in a buffer containing 100mM-KCl and 20mM-Tris/HCl, pH 7.4, by measuring the rate of NADH oxidation on the addition of 1mM-oxaloacetate. For the assay of ornithine transcarbamoylase, the fractions were incubated in a medium containing 100mM-KCl, 20mM-Tris/HCl, 50mM-ornithine hydrochloride and 10mM-carbamoyl phosphate at pH 7.4. Incubations were terminated by the addition of HC104 as described above, and citrulline was determined in the supernatant by the method of Archibald (1944). All enzyme assays were linear with time and protein concentration under the conditions employed. Protein was measured by a biuret method (Gornall et al., 1949), with bovine serum albumin as standard.

Results

Properties of glutaminase in mitochondria disrupted by freezing and thawing

It has been shown that, in intact liver mitochondria, glutaminase activity is stimulated in the presence of phosphate by the addition of NH4+ (Charles, 1968; Joseph & McGivan, 1978a,b) or HCO3− ions (Joseph & McGivan, 1978b), and this activation is to some extent dependent on the energy state of the mitochondria. It was also noted that NH4+ and HCO3− ions together with phosphate stimulated glutaminase also in disrupted mitochondria, and this stimulation was further increased in the presence of ATP.

These results are confirmed and extended in Fig. 1, where the effect of these activators on glutaminase activity in frozen and thawed mitochondria is shown. The concentration of phosphate required for half-maximal activity in the absence of other activators was approx. 25 mM, and this was decreased to 4–6mM in the presence of HCO3− and NH4+ ions. These ions did not greatly increase

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Fig. 1. Dependence of glutaminase activity in frozen and thawed mitochondria on the concentration of added phosphate

The experiment was performed as described in the Materials and methods section. The incubation contained 30mM-glutamine, 10mM-Tris/HCl, 4–6mg of mitochondrial protein and (a) no further addition, (b) 20mM-KHCO3, (c) 2mM-NH4Cl or (d) 20mM-KHCO3 plus 2mM-NH4Cl. ○, Additions as described above; O, as above plus 2mM-ATP and oligomycin (5μg/ml).
glutaminase activity in the presence of saturating phosphate concentrations. In the absence of these activators, ATP decreased the phosphate requirement for half-maximal activity to 12 mM.

When ATP was added in the presence of either \( \text{NH}_4^+ \) or \( \text{HCO}_3^- \) ions, a large activation was observed even in the absence of added phosphate, and the apparent maximal velocity of the enzyme was increased. Although the mechanism by which activation occurs is not at present understood, it appears that \( \text{NH}_4^+ \) and \( \text{HCO}_3^- \) ions exert their effect by decreasing the requirement of the enzyme for phosphate. Activation by ATP, on the other hand, is relatively independent of phosphate concentration, but requires the presence of \( \text{NH}_4^+ \) or \( \text{HCO}_3^- \) ions. It should be noted that in Fig. 1(a) \( \text{NH}_4 \) is continually produced as a result of glutaminase activity, and this may account for the observed decrease in phosphate requirement on the addition of ATP.

The concentrations of \( \text{NH}_4^+ \) and \( \text{HCO}_3^- \) ions required for half-maximum effects were found to be 0.2 and 4 mM respectively. These concentrations are similar to those required for half-maximum effects in intact mitochondria (Joseph & McGivan, 1978a). The concentration of ATP required to give half the maximum rate in the presence of 20 mM-\( \text{HCO}_3^- \) and absence of phosphate in disrupted mitochondria was found to be 1 mM.

As shown in Fig. 2, the glutamine-concentration-dependence is highly sigmoidal in the presence of 100 mM-phosphate. This sigmoidicity was decreased in the presence of \( \text{NH}_4^+ \) ions and abolished in the presence of \( \text{HCO}_3^- \). This is reflected in the concentrations of glutamine required for half-maximum activity (Table 1). The maximum activity of glutaminase assayed in the presence of 100 mM-glutamine and 150 mM-phosphate was not greatly affected by pH above pH 7.3, but below this the activity decreased sharply (Fig. 3). It is likely that this curve represents the true pH-dependence of the enzyme, since the phosphate concentration used is sufficient to saturate the enzyme throughout this pH range.

**Distribution of glutaminase after freezing and thawing or sonication of mitochondria**

The intramitochondrial location of glutaminase was investigated by disrupting the mitochondria by freezing and thawing or by sonication and separating the membrane and soluble fractions by centrifugation at 40,000 g for 30 min. Table 2 shows the percentages of glutaminase and marker-enzyme activities in the pellet fraction. The recovery of activity was at least 85% throughout.

Disruption of mitochondria by freezing and thawing three times caused the release of 50–60% of the matrix enzymes citrate synthase, malate dehydrogenase and ornithine transcarbamoylase. It is possible that some of these soluble enzymes were

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**Fig. 2. Dependence of glutaminase activity in frozen and thawed mitochondria on the concentration of added glutamine**

Disrupted mitochondria were incubated with 10 mM-Tris/HCl, pH 7.4, various concentrations of glutamine and activators as shown: \( \triangle \), 100 mM-potassium phosphate; \( \bigcirc \), 20 mM-potassium phosphate + 2 mM-NH\(_4\)Cl + 2 mM-ATP + oligomycin (5 \( \mu \)g/ml); \( \bigcircle \), 20 mM-potassium phosphate + 20 mM-KH\(_2\)PO\(_4\).

**Fig. 3. pH-dependence of glutaminase activity in frozen and thawed mitochondria**

The incubation medium contained 100 mM-glutamine, 150 mM-potassium phosphate, 10 mM-Tris/HCl and 3 mg of mitochondrial protein/ml.
trapped in vesicles in the pellet fraction. Glutamate dehydrogenase, which is also generally regarded as a matrix enzyme, was consistently found not to be released to the same extent. Under these conditions, glutaminase was found almost entirely in the pellet fraction, and further freezing and thawing did not increase the release of this enzyme.

When the mitochondria were disrupted by sonication, more protein was found in the soluble fraction. Approx. 40% of the inner-membrane marker enzyme succinate–cytochrome c reductase was sedimented at 40000g, but very little of the matrix enzymes or glutamate dehydrogenase were found in the pellet fraction. Under these conditions, glutaminase did not sediment to any extent. Further centrifugation of the soluble fraction at 100000g for 30 min caused complete sedimentation of succinate–cytochrome c reductase, but no further sedimentation of either glutaminase or any of the other marker enzymes (results not shown). It appears that in intact mitochondria, glutaminase is loosely associated with the inner membrane; disruption of the mitochondria by the mild procedure of freezing and thawing does not detach the enzyme from the membrane, but more vigorous treatment by sonication completely solubilizes it.

Differences in the characteristics of glutaminase activity between frozen-and-thawed and sonicated mitochondria

The ‘membrane-bound’ and ‘soluble’ forms of glutaminase were investigated with the frozen-and-thawed pellet fraction and the sonicated supernatant

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<tr>
<th>Table 1. Concentrations of glutamine required for half-maximal activity of glutaminase in frozen and thawed rat liver mitochondria</th>
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<tr>
<td><strong>Additions</strong></td>
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<td>20 mM-phosphate + 20 mM-KHCO₃</td>
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<tr>
<td>20 mM-phosphate + 20 mM-KHCO₃ + 2 mM-ATP</td>
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<tr>
<td>20 mM-phosphate + 20 mM-KHCO₃ + 2 mM-NH₄Cl</td>
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<td>20 mM-phosphate + 20 mM-KHCO₃ + 2 mM-ATP</td>
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<td>100 mM-phosphate</td>
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<td>20 mM-phosphate + 20 mM-KHCO₃ + 2 mM-NH₄Cl + 2 mM-ATP</td>
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Fig. 4. Arrhenius plots of the temperature-dependence of the membrane-bound and soluble forms of glutaminase. The incubation medium contained 100 mM-glutamine, 150 mM-potassium phosphate and 10 mM-Tris/HCl at pH 7.4. 0, Sonicated supernatant (soluble form); ●, frozen and thawed pellet (membrane-bound form). Activation energies were calculated to be (a) 97.7 kJ/mol, (b) 18.3 kJ/mol, (c) 52.0 kJ/mol. Under these conditions, the rate approximates to $V_{max}$ (nmol/min per mg).

<table>
<thead>
<tr>
<th>Table 2. Distribution of enzymes after freezing and thawing or sonicating rat liver mitochondria and sedimenting at 40000g for 30 min</th>
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<tbody>
<tr>
<td>Enzyme</td>
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<tr>
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</tr>
<tr>
<td>Glutaminase</td>
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<tr>
<td>Succinate–cytochrome c reductase</td>
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<td>Malate dehydrogenase</td>
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<td>Glutamate dehydrogenase</td>
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<td>Citrate synthase</td>
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<td>Ornithine transcarbamoylase</td>
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fraction respectively. The glutamine-concentration-dependences of these two forms were assayed in the presence of 20 mM-phosphate plus 20 mM-bicarbonate. The apparent $K_m$ for glutamine of the membrane-bound form was $6.18 \pm 0.32$ mM (mean $\pm$ S.E.M. for five preparations) and for the soluble form was $21.0 \pm 1.4$ mM (mean $\pm$ S.E.M. for four preparations). A previous report indicated that the $K_m$ of isolated rat liver glutaminase is 28 mM (Huang & Knox, 1976), and this probably corresponds to the 'soluble' form of the enzyme in the present investigation. The activation of the soluble form of glutaminase by ATP, HCO$_3^-$, phosphate and NH$_4^+$ ions (results not shown) showed similar characteristics to those exhibited by the membrane-bound form (see Fig. 1).

Arrhenius plots of the temperature-dependences of the membrane-bound and soluble forms of glutaminase are presented in Fig. 4. The plot for the membrane-bound form showed a break at 23°C, whereas that for the soluble form showed no such discontinuity.

Although incomplete release of cytosolic enzymes on freezing and thawing can be attributed to vesicle formation, it is unlikely that any significant permeability barrier to substrates exists since $[^{14}]$C-sucrose penetrates frozen and thawed mitochondria to the same extent as does $^3$H$_2$O (results not shown). There is no indication that glutamine transport limits glutaminase activity in intact mitochondria (Joseph & McGivan, 1978b). Thus none of the changes in temperature-dependence, concentration-dependence or enzyme distribution should be attributable to differential permeability barriers to glutamine.

**Discussion**

The results in the present paper indicate that glutaminase is loosely bound to the inner mitochondrial membrane. Membrane attachment is deduced from the distribution of the enzyme on freezing and thawing the mitochondria and from the temperature-dependence data. A break in the Arrhenius plot has been considered to be due to the phospholipid phase changes, which affect the activity of membrane-bound enzymes (Lenaz et al., 1972; Raison et al., 1971). It has been reported that loosely bound as well as integral proteins are affected in this way (Matlib & O'Brien, 1975). Kovacevic (1976) has previously shown that the Arrhenius plots obtained for glutaminase in frozen and thawed kidney mitochondria exhibit a break at 26°C.

In frozen and thawed liver mitochondria, glutaminase activity is modified by the presence of phosphate, ATP, NH$_4$Cl and KHCO$_3$, in accordance with previous findings (Joseph & McGivan, 1978b). NH$_4^+$ and HCO$_3^-$ ions decrease the requirement of the enzyme for phosphate, whereas ATP increases the rate at all phosphate concentrations in the presence of either NH$_4^+$ or HCO$_3^-$ ions. Activation of the enzyme by these compounds is likely to be of importance in the regulation of this enzyme in the cell. However, the mechanism by which these compounds exert their specific effects on liver glutaminase is not at present understood. Effects of HCO$_3^-$ and ATP on kidney glutaminase have not been reported; NH$_4^+$ ions activate this enzyme only at high concentrations (Kvamme et al., 1970).

On sonication of liver mitochondria, glutaminase is detached from the membrane, as judged by fractionation studies and the absence of a break in the Arrhenius plot. This is consistent with the findings of Kalra & Brosnan (1973), who used sonication or detergents to disrupt the mitochondria; Kovacevic (1976) obtained similar results with glutaminase in sonicated or detergent-treated kidney mitochondria. Sonication does not abolish the break in the Arrhenius plot obtained for integral proteins such as succinate dehydrogenase (Raison et al., 1971), and this reinforces the conclusion that glutaminase in liver is not an integral protein but is loosely bound to the mitochondrial membrane. The apparent $K_m$ for glutamine of solubilized or isolated glutaminase is much higher than that for the enzyme in frozen and thawed mitochondria. It is therefore clear that attachment of the enzyme to the membrane is important in the regulation of its activity. Studies on the isolated liver enzyme may therefore be of limited application in elucidating the regulation of glutaminase in the intact cell.

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**References**


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