The stimulation of glutamine hydrolysis in isolated rat liver mitochondria by Mg^{2+} depletion and hypo-osmotic incubation conditions

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1. In respiring rat liver mitochondria EDTA stimulates glutaminase activity measured in the presence of phosphate and HCO_3^- ions. The stimulation can be reversed by the addition of low concentrations of MgCl_2 . EGTA does not stimulate glutamine hydrolysis. 2. Glutaminase activity assayed in disrupted mitochondria is not significantly affected by EDTA or MgCl_2 . 3. The addition of EDTA results in a decrease in the concentration of phosphate required for half-maximal glutaminase activity. 4. Depletion of mitochondrial Mg^{2+} by the addition of the ionophore A23187 also stimulates glutamine hydrolysis in both the presence and the absence of EDTA. The effect of the ionophore can be abolished by the addition of MgCl_2 . 5. Hypo-osmotic incubation conditions increase the rate of mitochondrial glutamine hydrolysis. The effect of hypo-osmoticity on glutaminase is much less when EDTA is present. 6. It is suggested that glutaminase is partially and indirectly inhibited by endogenous mitochondrial Mg^{2+} and that the inner membrane may play a role in the regulation of glutaminase activity.

Glucagon has been shown to stimulate gluconeogenesis from glutamine to a greater extent than from any other amino acid tested (Joseph & McGivan, 1978a). Low concentrations of NH_4Cl have also been found to stimulate glutamine metabolism in the perfused liver (Chamalaun & Tager, 1970; Häussinger *et al.*, 1975; Häussinger & Sies, 1979) and in isolated liver cells (Joseph & McGivan, 1978a). Evidence has been presented to suggest that both these activators increase the effective activity of mitochondrial phosphate-dependent glutaminase (EC 3.5.1.2) (Joseph & McGivan, 1978a). More recently leucine has been shown to stimulate glutamine metabolism in isolated liver cells, and the effect of this activator has also been attributed to a stimulation of mitochondrial glutaminase (Baverel & Lund, 1979).

The regulatory properties of mitochondrial glutaminase are complex. Glutamine hydrolysis in intact mitochondria is stimulated by NH_4Cl (Charles, 1968; Joseph & McGivan, 1978b) and by HCO_3^- ions (Joseph & McGivan, 1978b). Glutamine metabolism in isolated liver cells has also been shown to be HCO_3^- -dependent (Baverel & Lund, 1979). Maximal rates of glutamine hydrolysis, in the presence of saturating concentrations of NH_4^+

or HCO_3^- , are obtained only on addition of a respiratory substrate.

It has now been found that the addition of EDTA to isolated intact mitochondria stimulates glutaminase activity assayed in the presence of either NH_4^+ or HCO_3^- ions. In addition, hypo-osmotic incubation conditions also increased glutaminase activity markedly. The characteristics of both these effects have been investigated in the present paper.

Materials and methods

Isolation of mitochondria

Liver mitochondria were isolated from fed male Wistar rats, weighing 200–250 g, by the method of Hogeboom (1955) as modified by Myers & Slater (1957). The isolation medium contained 200 mM-mannitol, 10 mM-Tris/HCl (pH 7.1) and 1 mM-Tris/EGTA. The mitochondria were used within 1 h of preparation.

Assay of glutaminase activity and incubation conditions

Glutaminase activity in mitochondria was measured as glutamate production from added glutamine under conditions where glutamate oxi-

dation was prevented by the addition of rotenone (Joseph & McGivan, 1978b). Unless otherwise stated the mitochondria were incubated in an air-saturated medium (pH 7.2–7.4) containing 15 mM-L-glutamine, rotenone (5 µg/ml), 10 mM-Tris/succinate, 10 mM-Tris/phosphate, 20 mM-Tris/HCl and either 100 mM-KCl (KCl medium) or 200 mM-mannitol (mannitol medium). Where HCO_3^- was added, either as the K^+ or the choline salt, the medium was gassed with O_2/CO_2 (19:1).

Incubations (final volume 1 ml) were carried out in 25 ml glass scintillation vials in a shaking water bath at 30°C. After 10 minutes the incubations were stopped by the addition of HClO_4 (final concn. 3.5%, w/v). After centrifugation (10000 g for 2 min) and removal of the precipitated protein, the supernatant was neutralized with 5 M-KOH/0.3 M-4-morpholinepropanesulphonate (K^+ salt). Glutamate was assayed enzymically by the method of Bernt & Bergmeyer (1965). After a short lag of 1–2 min, glutamate production was linear for up to 10 min in the presence or absence of EDTA (results not shown). Protein was determined by a biuret method (Gornall *et al.*, 1949), standardized with bovine serum albumin. Phosphate was measured by the method of Eibl & Lands (1969).

Where Mg^{2+} concentrations were varied, the following experimental protocol was used. Stock solutions of 200 mM-EDTA and of 200 mM-EDTA containing 200 mM- MgCl_2 were prepared. No further proton release was observed on addition of MgCl_2 or EDTA to the latter solution, indicating that MgCl_2 and EDTA were present in stoichiometric amounts. Amounts of both solutions were added to the incubation such that the MgCl_2 concentration was varied, but the EDTA concentration was constant at 2 mM.

Mitochondria were disrupted by six cycles of sonication (each 15 s) with a MSE sonicator with a 3 mm probe at a frequency of 20 kHz and an amplitude of 4 µm. These sonication conditions were found to inhibit the ADP-stimulation of succinate

respiration by 75–100%. Under these conditions glutaminase remains associated with membrane fragments (see McGivan *et al.*, 1980).

Results

Effect of EDTA on glutaminase

Isolated rat liver mitochondria, respiring on succinate (in the presence of rotenone), hydrolyse glutamine at a low rate (Table 1). In agreement with previous studies, it was found that this rate could be stimulated by the addition of HCO_3^- ions (Joseph & McGivan, 1978b). In the presence of 20 mM- KHCO_3 , the addition of 2 mM-EDTA resulted in a marked increase in the rate of glutamine hydrolysis. This effect was observed at all the concentrations of glutamine tested (results not shown). The addition of 2 mM-EGTA under identical conditions had no significant effect. It must be noted that 0.25 mM-EGTA, derived from the preparation medium, was present in all the incubations. No significant differences in the ability of EDTA to stimulate glutaminase could be demonstrated between these mitochondria and those prepared in a medium from which EGTA had been omitted (results not shown).

The dependence of the stimulation of glutamine hydrolysis on the concentration of added EDTA is shown in Fig. 1. In KCl medium, half-maximal stimulation of the hydrolysis of 15 mM-glutamine could be obtained by the addition of 58 ± 6 nmol of EDTA/mg of protein (mean $\pm$ S.E.M. of four mitochondrial preparations). Fig. 1 also shows that disruption of the mitochondria by mild sonication greatly decreased the ability of EDTA to stimulate glutaminase. This was also noted when the mitochondria were disrupted by freeze-thawing. The addition of concentrations of MgCl_2 up to 5 mM did not significantly influence glutaminase activity in freeze-thawed or sonicated mitochondria (results not shown). Added ATP has been shown to stimulate glutamine hydrolysis in disrupted mitochondria (Joseph & McGivan, 1978b). However,

Table 1. *Effect of EDTA on glutaminase activity in intact mitochondria*

Glutamate production from added glutamine was measured in either KCl medium or mannitol medium, whose compositions are given in the Materials and methods section. HCO_3^- was added as the potassium salt in the KCl medium and as the choline salt in the mannitol medium. All results are the means $\pm$ S.E.M. for the numbers of observations shown in parentheses. Mitochondria were added at 3–5 mg/ml. * $P < 0.01$; †, no significant difference from the activity measured in the presence of glutamine and HCO_3^- .

Additions	Glutaminase activity (nmol/10 min per mg)	
	KCl medium	Mannitol medium
Glutamine (15 mM)	20 $\pm$ 2 (15)	15 $\pm$ 3 (10)
Glutamine + HCO_3^- (20 mM)	163 $\pm$ 23 (12)	123 $\pm$ 23 (10)
Glutamine + HCO_3^- + EDTA (2 mM)	348 $\pm$ 23 (12)*	245 $\pm$ 27 (9)
Glutamine + HCO_3^- + EGTA (2 mM)	208 $\pm$ 30 (6)†	—

Fig. 1. *Dependence of mitochondrial glutaminase activity on the concentration of added EDTA*

Intact (●) or sonicated (○) mitochondria were incubated at a concentration of 3.0mg/ml in KCl medium as described in the Materials and methods section; 20mM-KHCO₃ was present in all incubations.

Fig. 2. *Dependence of glutaminase activity on phosphate concentration*

Glutamate production from 15mM-glutamine was measured at various concentrations of phosphate in the presence (○) or absence (●) of 2mM-EDTA in KCl medium, whose composition is given in the Materials and methods section; 20mM-KHCO₃ was present in all the incubations. Mitochondria were added at 3.0mg/ml.

total mitochondrial ATP concentrations were not altered on addition of EDTA (results not shown).

EDTA is a better chelator of Mg²⁺ than is EGTA, but both chelators have similar dissociation constants for Ca²⁺ (Schmid & Reilly, 1957). The observation that only EDTA is effective in stimulating glutaminase suggests that the effect of this chelator is due to the removal from the mitochondria of Mg²⁺ and not of Ca²⁺. It was found that the stimulating effect of 2mM-EDTA was decreased by 90% on addition of 0.5mM-MgCl₂, and completely abolished by 1.0mM-MgCl₂ (results not shown). This suggests that the Mg²⁺ is being removed from a mitochondrial site which has a very high affinity for this bivalent cation.

The dependence of mitochondrial glutamine hydrolysis on the concentration of added phosphate at sub-saturating concentrations of glutamine is shown in Fig. 2. In the absence of any added phosphate, a very low rate of glutamine hydrolysis was observed. This shows that contamination of the mitochondrial preparation by 'phosphate-independent glutaminase' is negligible and also that HCO₃⁻ alone does not replace the phosphate requirement of the enzyme. The addition of EDTA greatly decreased the concentration of phosphate required for half-maximal stimulation of glutaminase from 12.1 ± 2.1

to 2.1 ± 0.2 mM (mean ± S.E.M. of three experiments) when the activity was measured in respiring mitochondria incubated in a KCl medium with 15mM-glutamine and 20mM-KHCO₃. Under these conditions the maximal rate was slightly increased by EDTA from 238 ± 15 to 287 ± 14 nmol of glutamate/10min per mg of protein. It seems from these data that the chelation of endogenous Mg²⁺ greatly increases the sensitivity of glutaminase for phosphate. This may be brought about either by a conformational change in the enzyme molecule or by increasing the concentration of phosphate available to the enzyme. Direct measurements of the concentration of phosphate in the mitochondrial matrix after centrifugation of the mitochondrial suspension through silicone oil indicated that after 2min incubation with an external phosphate concentration of 4mM the internal concentration, after corrections for adhering phosphate in the pellet, was increased by EDTA from 17 to 22 mM (mean of two experiments). It is unlikely that this increase in the mitochondrial phosphate concentration could by itself account for the 3–4-fold activation of glutaminase by EDTA observed under these conditions (see Fig. 2).

Effect of the bivalent-cation ionophore A23187

The endogenous Mg^{2+} content of mitochondria is approx. 30 nmol/mg of protein (Johnson & Press-

Fig. 3. Effect of ionophore A23187 on glutaminase activity

Glutamate production from 15 mM-glutamine was measured in the presence of various concentrations of ionophore A23187 in a medium containing 200 mM-mannitol without $MgCl_2$ (a) or with 2 mM- $MgCl_2$ (b). The solvent for the ionophore was dimethyl sulphoxide, present at 0.7% (v/v) in all the incubations. ▲, No added HCO_3^- ; ●, +20 mM-choline bicarbonate; ○, +20 mM-choline bicarbonate + 2 mM-EDTA. Mitochondria were added at 3 mg/ml.

man, 1969; Diwan *et al.*, 1979). Bogucka & Wojtczak (1971) have shown that part of this Mg^{2+} is located outside the inner membrane of the mitochondria. It is known that EDTA does not penetrate the inner membrane of the mitochondria at neutral pH (Settlemyre *et al.*, 1968; Caswell, 1972; Wehrle *et al.*, 1976). It follows therefore that the stimulatory effect of EDTA on glutamine hydrolysis in intact mitochondria is due to the chelation of Mg^{2+} present outside the inner membrane. The bivalent-cation ionophore A23187 increases the permeability of the inner membrane for Mg^{2+} and Ca^{2+} ions (see Reed & Lardy, 1972; Wong *et al.*, 1973). The possible role of the internal pool of Mg^{2+} was investigated by using this ionophore.

The addition of ionophore A23187 to respiring mitochondria incubated in mannitol medium with 15 mM-glutamine and 20 mM- HCO_3^- resulted in a marked stimulation of glutamine hydrolysis (Fig. 3a). When EDTA was added to stimulate glutaminase activity, the addition of the ionophore resulted in a further increase in enzyme activity. These results suggest that depleting either the internal or the external pools of Mg^{2+} stimulates glutaminase activity as expressed in intact mitochondria.

Fig. 3(b) shows that the addition of 2 mM- $MgCl_2$ prevented the stimulatory effect of both EDTA and ionophore A23187. When both of these activators were present together, the addition of $MgCl_2$ prevented the effect of EDTA, but not that of the ionophore. One possible interpretation of these findings is that the ionophore activates glutaminase by depleting the internal pool of Mg^{2+} ; this depletion can only occur when the external free concentration of Mg^{2+} is less than the internal free concentration, which has been estimated to be 1.4 mM (Williamson *et al.*, 1979). When the external concentration of Mg^{2+} is 2.0 mM, no depletion of Mg^{2+} occurs, and the ionophore does not stimulate glutaminase activity. When 2 mM- Mg^{2+} plus 2 mM-EDTA is present, the free external concentration can be calculated to be not greater than 40 μM , and under these conditions the ionophore depletes the internal pool of Mg^{2+} , resulting in a stimulation of glutaminase activity. The stimulation of glutamine hydrolysis by ionophore A23187 and its reversal by added $MgCl_2$ was found to occur in both the presence and the absence of 2 mM-EGTA (results not shown). This is not consistent with a major regulatory role of Ca^{2+} on glutamine hydrolysis.

Effect of changes in osmolarity of the incubation medium on glutaminase activity in intact mitochondria

The mechanism by which EDTA stimulates glutaminase must be indirect, since the effect is

Fig. 4. Effect of changes in osmolarity on glutaminase activity

Glutamate production from 15 mM-glutamine was measured in a medium containing 10 mM-succinate, 20 mM-Tris/HCl, 10 mM-Tris/phosphate, 20 mM-choline bicarbonate, rotenone (5 μ g/ml) and various concentrations of KCl. ●, No EDTA; ○, +2 mM-EDTA. Mitochondria were added at 3 mg/ml.

observed in intact and not in disrupted mitochondria (Fig. 1). It is known that the addition of EDTA to respiring mitochondria incubated in the presence of a permeant anion and a univalent cation results in swelling of the mitochondria (Azzone & Azzi, 1966; Azzi *et al.*, 1966; Settlemire *et al.*, 1968; Wehrle *et al.*, 1976; Blair, 1977, 1979). The effect of changes in the mitochondrial matrix volume on glutaminase activity was investigated by varying the osmolarity of the incubation medium with KCl. Fig. 4 shows that the rate of glutamine hydrolysis, measured in the presence of HCO_3^- ions, was greatly increased by decreasing the osmolarity of the incubation medium. Qualitatively similar results were obtained when choline chloride or mannitol was used to adjust the osmolarity (results not shown). A much smaller change in glutaminase activity owing to change in osmolarity was observed

when 2 mM-EDTA was present in the incubation medium. The ratio of the rates of glutamine hydrolysis measured at 155 mosM (no added KCl) and 355 mosM (100 mM-KCl) under the conditions shown in Fig. 4 was 5.0 ± 0.9 in the absence of EDTA and 1.4 ± 0.1 in the presence of EDTA (mean $\pm$ S.E.M. of four experiments).

NH_4Cl has been shown to stimulate glutamine hydrolysis in isolated mitochondria (Charles, 1968; Joseph & McGivan, 1978b). Both EDTA and hypo-osmotic treatment stimulated glutaminase activity when measured in the presence of 5 mM- NH_4Cl , and the effect of osmolarity was greatly decreased when EDTA was present in the incubation medium (results not shown).

Discussion

The results reported in this paper show that glutaminase activity assayed in intact respiring mitochondria, in the presence of phosphate and HCO_3^- , can be further increased by the addition of EDTA or ionophore A23187, or by decreasing the osmolarity of the incubation medium.

The ability of EDTA to induce mitochondrial swelling, as judged by light-scattering measurements, has long been known. In an early investigation Guha & Chakravati (1960) reported a correlation between the changes in the light-scattering by mitochondria and glutaminase activity. These workers concluded that part of the phosphate-dependence of glutaminase may reflect the role of this anion in stimulating swelling. They suggested that the stimulation of glutaminase by mitochondrial swelling may be due to an increase in the permeability of the mitochondria for glutamine. It is therefore necessary to consider the possibility that the activation of glutaminase by EDTA is also a result of an increased swelling of the mitochondria in the presence of this metal chelator.

The possibility that the EDTA stimulation of glutaminase may be related to an increase in mitochondrial swelling is suggested by the observation that mitochondrial swelling, like glutaminase activity, is stimulated by EDTA and not by EGTA, and that the effects of EDTA on mitochondrial swelling and glutaminase activity are reversed by the addition of less than stoichiometric amounts of Mg^{2+} (Azzi *et al.*, 1966).

A tightly bound pool of membrane Mg^{2+} is thought to regulate the permeability of the inner membrane to univalent cations (Brierley, 1976). The effect of EDTA on light-scattering requires the presence of univalent cations in the incubation medium. In agreement with these observations, we found no effects on light-scattering on addition of

EDTA or ionophore A23187 to mitochondria suspended in mannitol medium under the conditions shown in Table 1 or Fig. 3 (results not shown). Yet under both these conditions glutaminase activity is increased. It must therefore be concluded that the mere removal of mitochondrial Mg^{2+} from inside the inner membrane (by the addition of ionophore A23187) or outside the inner membrane (by the addition of EDTA) results by itself in an activation of glutaminase, and is not secondary to a change in permeability of the inner membrane to univalent cations or to an increase in the mitochondrial volume.

The question arises of the mechanism by which mitochondrial Mg^{2+} regulates glutaminase activity. A possible explanation of the activating effect of EDTA could be that it chelates membrane-bound Mg^{2+} , resulting in structural changes in the membrane, which in turn alter the activity of enzymes, such as glutaminase, that are associated with the inner membrane (McGivan *et al.*, 1980).

It is noteworthy that glutamine hydrolysis was stimulated by hypo-osmotic incubation conditions in the absence of EDTA, but much less in its presence (Fig. 4). This suggests that the mechanism of action of EDTA and hypo-osmoticity may be related. A possible mechanism of this effect is that the passive movement of water into the mitochondria, and the accompanying unfolding of the cristae, result in changes in the enzyme-membrane interaction identical with that produced by EDTA. Another possibility is that hypo-osmotic conditions may dilute the matrix pool of Mg^{2+} , causing a redistribution of this ion within the mitochondria such that the amount of membrane-bound Mg^{2+} is decreased. Hypo-osmotic incubation conditions have also been shown to stimulate glutamine hydrolysis in kidney mitochondria (Welbourne *et al.*, 1976; Kovačević *et al.*, 1979), where the enzyme is also associated with the inner membrane (Curthoys & Weiss, 1974; Kovačević, 1976).

Previous studies have shown that glutamine equilibrates very rapidly across the mitochondrial inner membrane (Joseph & McGivan, 1978b), and it is therefore unlikely that the activation of glutaminase by Mg^{2+} depletion and hypo-osmotic incubation conditions is due to an increased transport of glutamine into the mitochondria. However, an effect on glutamate efflux cannot be excluded.

The activation of glutaminase by EDTA in intact mitochondria results in a decrease in the concentration of phosphate required for half-maximal activity of the enzyme (Fig. 2). The addition of the activators, NH_4^+ or HCO_3^- ions, to freeze-thawed mitochondria also results in a decrease in the phosphate concentration required for half-maximal glutaminase activity (McGivan *et al.*, 1980). It would seem that changes in the phosphate-con-

centration-dependence of the enzyme may be of significance in the regulation of this enzyme in the intact cell.

The free concentrations of Mg^{2+} in both the cytosol and mitochondrial compartments of the liver cell are relatively high (Veloso *et al.*, 1973; Walajtys *et al.*, 1973; Van der Meer *et al.*, 1978). It is important to stress that glutamine hydrolysis in intact mitochondria is activated by HCO_3^- or NH_4^+ even in the presence of added Mg^{2+} . Full expression of glutaminase activity, in the presence of HCO_3^- or NH_4^+ , requires the removal of part of the endogenous mitochondrial Mg^{2+} . A similar effect on mitochondrial Mg^{2+} in the intact cell could stimulate flux through glutaminase. However, the physiological conditions that lead to changes in mitochondrial Mg^{2+} , and the precise role played by Mg^{2+} in the regulation of glutaminase activity, remain to be established.

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