Influence of phospholipids on the activity of phosphate-dependent glutaminase in extracts of rat liver mitochondria

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Liver glutaminase can be solubilized from frozen-and-thawed mitochondria by treatment with phospholipase A₂. Solubilization by this technique markedly changes the kinetic properties of the enzyme. The properties of the membrane-bound form of the enzyme are partially restored by adding phosphatidylcholine or phosphatidylethanolamine to the phospholipase extract. It is concluded that the kinetic properties of liver glutaminase are a function of the interaction of this enzyme with membrane phospholipids.

Liver phosphate-dependent glutaminase (EC 3.5.1.2) is a mitochondrial enzyme. The effective activity of this enzyme in isolated intact liver mitochondria is dependent on the mitochondrial energy state (Joseph & McGivan, 1978), and is increased by mitochondrial swelling in hypo-osmotic media and by treatment of the mitochondria with EDTA (Joseph *et al.*, 1981). Injection of glucagon into rats results in an increased effective glutaminase activity in subsequently isolated liver mitochondria (Lacey *et al.*, 1981). It has been shown also that addition of glucagon, adrenaline or vasopressin to isolated hepatocytes produced an increased glutaminase activity in mitochondria subsequently isolated from these cells (Corvera & Garcia-Sanchez, 1983). The molecular basis of this activation has not yet been elucidated.

On the basis of fractionation studies, it has been concluded that glutaminase is loosely bound to the mitochondrial inner membrane. Prolonged sonication of the mitochondria was found to solubilize the enzyme and to change its kinetic properties (McGivan *et al.*, 1980). However, no interaction of glutaminase with components of the mitochondrial membrane has yet been demonstrated. In the present paper, evidence is reported that the kinetic properties of liver glutaminase can be affected by certain phospholipids under appropriate experimental conditions.

Methods and materials

Rat liver mitochondria were prepared as described by Chappell & Hansford (1972) in a medium containing 0.25 M-mannitol, 10 mM-Tris/HCl and

1 mM-EGTA, pH 7.4. The mitochondrial suspension was diluted with an equal volume of 50 mM-Tris/HCl, pH 8.0, and was rapidly frozen and thawed three times. Phospholipase extracts of mitochondria were prepared as follows: to the frozen-and-thawed mitochondrial suspension was added 1 mM-CaCl₂ plus 5 units of phospholipase A₂/ml. The suspension was incubated at 30°C for 10 min and the phospholipase reaction was terminated by the addition of 10 mM-EGTA to remove the Ca²⁺ required as an obligatory activator for this enzyme. The suspension was centrifuged at 40 000 *g* for 20 min and the supernatant fraction was used as the phospholipase extract. The above incubation time, Ca²⁺ concentration and amount of phospholipase added were found to be optimal in this system for the release of glutaminase from the membrane fraction.

Glutaminase activity was measured by following the production of glutamate from added glutamine (McGivan *et al.*, 1980). The frozen-and-thawed mitochondrial suspension or phospholipase extract was incubated at a concentration of 5 mg of protein/ml with various concentrations of glutamine and phosphate, together with 2 mM-NH₄Cl (obligatory activator of glutaminase), and 50 mM-Tris/HCl at pH 8.0 and 30°C. The reaction was terminated by the addition of HClO₄ (3.5%, w/v) at various times. After centrifugation of the precipitated protein (10 000 *g* for 2 min), glutamate was assayed in the neutralized extract.

Lipids were extracted from ox liver mitochondria with a mixture of chloroform, methanol and water (Weinbach & Garbus, 1965). The lipids were separated by chromatography on DEAE-cellulose (Rouser & Fleischer, 1967). In reconstitution experiments, phospholipids were suspended in 50 mM-

Tris/HCl, pH 8.0, by gentle shaking. The suspension was then sonicated at 40 W with a Soni-probe (Dawe Instruments) until translucent.

Glutamate was assayed enzymically (Bernt & Bergmeyer, 1965). Phospholipids were digested with a mixture of HClO_4 and H_2SO_4 and assayed as P_i (Chen *et al.*, 1956). Protein was assayed by a biuret method (Gornall *et al.*, 1949). Phospholipase and purified phospholipids were purchased from Sigma.

Results

Effects of phospholipase A_2 on glutaminase activity

In frozen-and-thawed mitochondria, 89% of the glutaminase activity was recovered in the pellet fraction on centrifugation at 40 000 *g* for 10 min, in agreement with previous results (McGivan *et al.*, 1980). Treatment of the frozen-and-thawed mitochondrial suspension with phospholipase A_2 in the presence of Ca^{2+} led to the effective solubilization of the enzyme, and 88% of the recovered activity was found in the supernatant.

Fig. 1(a) shows the phosphate-dependence of glutaminase activity in frozen-and-thawed mitochondria and in a phospholipase extract of this

preparation. In frozen-and-thawed mitochondria at pH 8.0 and at 50 mM-glutamine, approx. 70% of the glutaminase activity was found to be independent of added phosphate. In contrast, in the phospholipase supernatant, the enzyme activity was completely dependent on added phosphate, with a half-maximum rate at 8 mM-phosphate. Incubation of the phospholipase extract with total lipids extracted from ox liver mitochondria caused an activation at all phosphate concentrations and produced a curve intermediate between those obtained for frozen-and-thawed mitochondria and for phospholipase extracts. The activation was maximal in the range 5–10 mM added phosphate.

Fig. 1(b) shows the dependence of glutaminase activity on glutamine concentration. Again in accordance with previous results (McGivan *et al.*, 1980), the glutamine-dependence in frozen-and-thawed mitochondria was hyperbolic, with a half-maximum velocity at approx. 8 mM. Treatment with phospholipase A_2 caused the concentration-dependence to become markedly sigmoidal, and the concentration of glutamine required for half-maximal velocity increased to 20 mM. The addition of mitochondrial lipids to the phospholipase-treated

Fig. 1. *Effect of phospholipase treatment and of re-addition of phospholipids on the activity of liver glutaminase* Various preparations of liver mitochondria as indicated were incubated at 30°C and pH 8.0 with 50 mM-Tris/HCl/2.5 mM- NH_4Cl and: (a) 50 mM-glutamate plus various concentrations of phosphate; (b) 50 mM-phosphate plus various concentrations of glutamine. Samples were taken at 10 and 20 min for low substrate concentrations or at 2 and 12 min for higher concentrations. The activity was taken as glutamate production at 20–10 or 12–2 min; the time course was linear over these intervals. The final protein concentration was 5 mg/ml. ●, Frozen-and-thawed mitochondria; ▲, phospholipase extract; ○, phospholipase extract plus 230 nmol of lipid P/mg of ox liver mitochondrial lipids.

supernatant activated at all glutamine concentrations and decreased the sigmoidicity of the concentration-dependence. Phospholipids were found to have no effect on glutaminase activity in frozen-and-thawed mitochondria which had not been treated with phospholipase.

Specificity of activation by phospholipids

To determine the component of the lipid mixture responsible for the activation of glutaminase, the mitochondrial lipids were separated by DEAE-cellulose chromatography, and each fraction was tested for its activating effect on glutaminase in a phospholipase extract in the presence of 50 mM-glutamine plus 5 mM-phosphate. Only those fractions containing phosphatidylcholine and phosphatidylethanolamine caused activation, and for a given lipid concentration phosphatidylcholine was more effective than phosphatidylethanolamine.

This phospholipid specificity was confirmed by using purified phospholipids obtained commercially. Fig. 2 shows that only phosphatidylcholine and phosphatidylethanolamine activated in this system. A half-maximal effect of phosphatidylcholine was obtained at a concentration of 18 nmol of lipid P/mg

Fig. 2. Specificity of activation of glutaminase by phospholipids

Phospholipase extract was incubated with 2.5 mM- NH_4Cl /50 mM-Tris/HCl/50 mM-glutamine plus 5 mM-phosphate at pH 8.0 and 30°C. Glutaminase activity was determined as glutamate production after 20 min minus that after 10 min. The protein concentration was 5 mg/ml and phospholipids were preincubated with the extract for 2 min before initiation of the reaction. ●, Phosphatidylcholine; ○, phosphatidylethanolamine; ▲, cardiolipin; △, phosphatidylserine.

of protein, whereas phosphatidylethanolamine was less effective, with a half-maximum requirement of 100 nmol of lipid P/mg. Cardiolipin and phosphatidylserine caused a small inhibition rather than an activation. The phosphate-dependence of the enzyme reconstituted with phosphatidylcholine (75 nmol/mg of protein) was very similar to that in the presence of ox mitochondrial lipids (see Fig. 1a).

In further experiments (results not shown), it was found that glutaminase activity in phospholipase extracts was not activated by 5 mM-phosphocholine, 5 mM-*O*-phospho-L-serine, 5 mM-*O*-phosphoethanolamine or 1 mM-L- α -phosphatidic acid. It is concluded that glutaminase is activated in this system only by phospholipid molecules having a positively charged group but no net negative charge. At pH 8, liver glutaminase binds strongly to DEAE-cellulose (J. D. McGivan & N. M. Bradford, unpublished work) and therefore is assumed to have a considerable net negative charge. Ionic interactions may therefore be important in the activation of this enzyme by phospholipids.

Discussion

The kinetic properties of liver glutaminase are considerably altered when this enzyme is detached from the mitochondrial membrane by treatment with phospholipase A_2 . The glutamine-concentration dependence becomes sigmoidal, and the enzyme becomes completely dependent on added phosphate. Since these changes in enzyme behaviour were partially reversed by the addition of phospholipids, it follows that the kinetic properties of the enzyme in frozen-and-thawed mitochondria are, at least in part, a function of the interaction of the enzyme with the membrane lipids. It has been shown previously that sonication 'solubilizes' glutaminase from mitochondria. In sonicated mitochondria, the glutamine-dependence is hyperbolic, although the K_m for glutamine increases from 6–8 mM in frozen-and-thawed mitochondria to 20 mM on sonication (McGivan *et al.*, 1980). It appears therefore that the frozen-and-thawed and phospholipase-treated mitochondria represent the fully membrane-associated and solubilized forms of glutaminase respectively, and sonicated mitochondria represent an intermediate state where the enzyme is still associated with the membrane lipids to some extent.

In phospholipase-treated extracts, glutaminase activity was not fully restored by the addition of phosphatidylcholine or ox liver phospholipids to that observed in frozen-and-thawed mitochondria. In particular, at low glutamine concentrations there was a considerable difference between the rates in frozen-and-thawed mitochondria and those in the reconstituted system (Fig. 1b). The glutamine content of freeze-clamped liver is 5–6 $\mu\text{mol/g}$

(Brosnan & Williamson, 1974). Even if this glutamine were all intracellular, the intramitochondrial concentration would be less than 10 mM (assuming no large concentration gradient across the mitochondrial membrane). Therefore at physiological glutamine concentrations interaction of glutaminase with phospholipids is not a complete explanation of the difference between the activity in frozen-and-thawed mitochondria and that in the phospholipase-treated extract. Glutaminase activity may also be affected by interaction of the enzyme with membrane proteins, and this interaction may be facilitated by the presence of phospholipids.

Mitochondria isolated from the livers of rats injected with glucagon exhibit an increased effective glutaminase activity, and this increased activity is associated with a decreased requirement for added phosphate (Lacey *et al.*, 1981). Mitochondria treated with EDTA also exhibit a decreased phosphate requirement for glutaminase activity (Joseph *et al.*, 1981). These effects are not due to an altered distribution of phosphate across the mitochondrial membrane. The results in the present paper indicate that the phosphate-dependence of glutaminase is dependent in part on the degree of association of this enzyme with membrane phospholipids. It is possible that stimulation of glutaminase activity by mitochondrial swelling and by glucagon is mediated via a modification in this lipid/protein interaction.

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