Activation of hepatic glutaminase by spermine

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Glutaminase activity in intact mitochondria from rat liver is activated by spermine, as indicated both by increased glutamate production from glutamine and by increased respiration with glutamine as sole substrate. Glutaminase activity, assayed in membranes from frozen–thawed mitochondria, is activated by spermine about 6-fold at physiological concentrations of its other effectors (NH_4^+ at 0.7 mM, P_i 5 mM) and at pH 7.4. Spermine decreased the apparent K_m for glutamine from 38 to

15 mM at 5 mM P_i , and increased the sensitivity of the enzyme for phosphate activation so that the concentration required for 50% stimulation decreased from 15 to 4 mM. Half-maximal spermine effects occurred at 0.15 mM, which is in the physiological range. Spermine was effective in the presence of physiological concentrations of Mg^{2+} . We suggest that spermine may be a physiological activator of hepatic glutaminase.

INTRODUCTION

Glutaminase, the principal enzyme of hepatic glutamine catabolism, is loosely attached to the mitochondrial inner membranes [1] of periportal hepatocytes [2]. It appears to be closely associated with enzymes of the urea cycle; specifically, ammonia produced by glutaminase becomes immediately available to carbamoyl phosphate synthetase [3]. Glutamine plays a key role in the nitrogen economy of animals. It is the amino acid present in the highest concentration in blood, is released in large amounts from skeletal muscle during starvation, serves as a major fuel for the intestine and for many growing cells, and, as an immediate precursor of urinary ammonia, plays a crucial role in acid–base homeostasis. Hepatic glutaminase is therefore a highly regulated enzyme (for a recent review see [4]) and, indeed, exercises very high control strength on hepatic glutamine utilization [5]. The absolute amount of glutaminase protein is determined by its relative rates of synthesis and degradation, and is known to vary under different physiological and pathological conditions [6]. The enzyme can also be regulated acutely. The hepatic isotype of glutaminase is activated by P_i and by its product, ammonia [7]. The physiological significance of activation by its product has been considered in the context of feed-forward activation of hepatic glutaminase by ammonia produced in the intestine [8]. Other activators include ATP, Mg^{2+} and citrate [1,9,10], but, because of the concentrations at which these are effective, they are unlikely to be of physiological significance. The reported apparent K_m values for glutamine vary widely, from 6 mM [1] to 44 mM [11], as does the nature of the dependence of velocity on substrate concentration, which can be either hyperbolic or sigmoidal. The principal determinants of the variation in the apparent K_m include factors such as association with the mitochondrial inner membrane, pH and the concentration of P_i . Although the reported apparent K_m values of glutaminase for glutamine are high, compared with the glutamine content of whole liver, which is reported to be about 5.0 $\mu\text{mol/g}$ [12], it is conceivable that the physiological concentration inside mitochondria is higher than that in the cytoplasm, since isolated rat liver mitochondria have been reported to concentrate glutamine [13], and indeed Häussinger et al. [14] reported the mitochondrial glutamine concentration to be 2–3 times the cytosolic concentration.

Glutaminase can be activated in hepatocytes and in the isolated

perfused liver by gluconeogenic hormones such as glucagon or adrenaline [15–17]. Indeed, liver mitochondria, isolated from rats injected with glucagon 25 min previously, displayed appreciably higher rates of glutaminase activity, provided that the mitochondria were intact. Increased glutaminase activity was not evident once the mitochondria were broken [18]. The mechanism of this hormonal activation has not been determined. We began a study of glutamine hydrolysis by isolated mitochondria, as a function of Ca^{2+} concentration, since hormones such as glucagon and adrenaline are known to elevate cytosolic Ca^{2+} [19,20]. We found that Ca^{2+} did activate glutamine hydrolysis in isolated mitochondria, but at concentrations (2–3 μM) which are quite supraphysiological. Since spermine stimulates Ca^{2+} uptake by rat liver mitochondria [21], we carried out experiments to see whether spermine would render glutaminase sensitive to physiological concentrations of Ca^{2+} . In the course of these experiments, we found that spermine itself, at very low concentrations, was a potent activator of glutaminase. This paper describes the effect of spermine on hepatic glutaminase.

MATERIALS AND METHODS

Liver mitochondria were isolated from adult male Sprague–Dawley rats (Charles River, Montreal, Canada) as previously described [22]. The mitochondria were suspended at a concentration of about 60 mg of protein/ml in the isolation medium (225 mM mannitol, 75 mM sucrose, 5 mM Hepes and 1 mM EGTA, pH 7.4). The respiratory control ratio of these mitochondria with α -oxoglutarate as substrate was greater than 6. Glutaminase activity in intact mitochondria was determined by the method of Kovacevic et al. [23]. The incubation medium (pH 7.4) contained 100 mM KCl, 10 mM Tris, 10 mM KH_2PO_4 , 10 mM KHCO_3 , 20 mM L-glutamine, 5 $\mu\text{g/ml}$ rotenone (to inhibit glutamate oxidation) and 5 mM succinate to energize the mitochondria. Incubations were carried out in plastic Erlenmeyer flasks (since polyamines bind readily to glass) for 10 min at 30 °C with a mitochondrial protein concentration of 1–4 mg/ml. The reaction was stopped with HClO_4 , neutralized with KOH, and the glutamate produced was measured by the method of Bernt and Bergmeyer [24]. Linearity of the assay with time and with protein concentration was determined in all cases. There was usually a lag period of about 2 min before rates became linear.

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Glutamine oxidation was assessed polarographically under State-3 conditions. The medium consisted of 100 mM KCl, 10 mM Tris/HCl, 10 mM K_2HPO_4 , 1 mM ADP, 0.52 mM EGTA and 25 mM L-glutamine.

Glutaminase activity was also determined in freeze-thawed mitochondria, prepared as described by Szveda and Atkinson [11]. Glutaminase was assayed at 37 °C in 300 mM mannitol/20 mM Hepes. Concentrations of K_2HPO_4 , NH_4Cl , glutamine and polyamine and the pH were varied as described in the Figure legends. Incubations, in triplicate, were for 10 min and contained 1 mg of mitochondrial protein/ml. Reactions were stopped with $HClO_4$, neutralized, and glutamate was measured [24]. In these assays, particular care was taken to ensure that the glutamine was minimally contaminated with ammonia. Stock glutamine solutions (200 mM) were prepared about 30 min before use and kept on ice. Immediately before beginning incubations, the solution was warmed to 37 °C. As with the mitochondrial assays, a zero-time value was subtracted from all measurements of glutamate. All assays were demonstrated to be linear with protein concentration and with time after a lag of about 2 min.

Stock polyamine solutions were neutralized and stored in polyethylene vials. NH_4Cl and $HClO_4$ were obtained from Fisher Scientific (Dartmouth, Nova Scotia, Canada). All other chemicals and enzymes were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

RESULTS

Spermine activates glutaminase in intact mitochondria

Figure 1 shows the effect of the three polyamines (putrescine, spermidine and spermine) on the glutaminase activity of intact mitochondria as determined by the method of Kovacevic et al. [23]. Spermine at 0.1 mM activated glutaminase about 5-fold. Activation by spermidine was also evident, but was somewhat less pronounced. In these experiments the mitochondria were preincubated with the polyamines for 6 min before addition of glutamine. Effects of polyamines were very much less in the

Figure 1 Effect of polyamines on glutaminase flux in intact rat liver mitochondria

Glutaminase assay was carried out as described in the Materials and methods section, by using 20 mM glutamine, 0.7 mM NH_4Cl and 5 mM phosphate, pH 7.4. Data represent means of three observations on separate mitochondrial preparations, and the error bar represents 1 S.E.M. The asterisk indicates a significant difference ($P < 0.05$, by paired t test) from the value with no added polyamine. Symbols: ■, spermine; ▲, spermidine; ●, putrescine.

Figure 2 Effect of spermine on respiration of rat liver mitochondria with glutamine as substrate

Glutamine oxidation was assessed polarographically under State-3 conditions, as described in the Materials and methods section. The arrows indicate addition of the following: M, mitochondria (1.0 mg of protein/ml); G, glutamine (25 mM); S, spermine (0.78 mM); A, ADP (0.52 mM).

absence of this preincubation, but complete activation was apparent after 6 min. We also examined the effect of spermine on the oxygen consumption of rat liver mitochondria under State-3 conditions. The data in Figure 2 show that addition of 0.8 mM spermine markedly stimulated the oxygen consumption of liver mitochondria with glutamine as sole substrate. The effect of spermine increased with preincubation (results not shown). These results suggest that spermine may be a potent activator of glutaminase. The effect of preincubation is consistent with a lag due to the need for the polyamine to penetrate the mitochondrial inner membrane. However, these experiments are not definitive in identifying glutaminase itself as the site of action of spermine, since other steps (glutamine entry into and glutamate exit from mitochondria in Figure 1, and glutamine entry and subsequent oxidation of the glutamate produced by glutaminase in Figure 2) could conceivably be limiting. It is likely that glutaminase itself is rate-controlling in both assays, since flux through both is stimulated by ammonia, an activator of glutaminase. When intact mitochondria were incubated under the conditions given in Figure 2, but with glutamate as sole substrate instead of glutamine, spermine had no effect on oxygen consumption (results not shown). Thus spermine does not affect the subsequent oxidation of glutamate. It was necessary, however, to examine the effects of the polyamines in a broken mitochondrial preparation, where permeability would not be an issue. We chose the freeze-thawed mitochondrial preparation described by Szveda and Atkinson [9] as most physiological, since the enzyme remains attached to the inner membrane.

Spermine activates glutaminase in broken mitochondria

In these experiments glutaminase activity was measured under near-physiological conditions. Except where specifically varied, the concentration of glutamine was 20 mM, that of P_i was 5 mM, that of NH_4^+ was 0.7 mM and the pH was 7.4. Results shown in Figures 3–8 are each from a single experiment which is typical of 3–5 similar experiments carried out on separate mitochondrial preparations. Figure 3 shows that both spermine and spermidine stimulated glutaminase. Spermine displayed the greatest (about 3-fold) effect, with a half-maximal effect at about 0.15 mM, but spermidine was also an effective activator. Preincubation with polyamines was not necessary in these experiments.

Figure 3 Effect of polyamines on glutaminase activity in freeze-thawed mitochondria from rat liver

Glutaminase was assayed in a medium containing 300 mM mannitol, 20 mM Hepes, pH 7.4, 20 mM glutamine, 5 mM phosphate and 0.7 mM NH_4Cl . Symbols: ■, spermine; ▲, spermidine; ●, putrescine.

Figure 4 Effect of NH_4^+ concentration on glutaminase activity in the absence and presence of spermine

Glutaminase activity was assayed in freeze-thawed mitochondria, in the presence of 300 mM mannitol, 20 mM Hepes, pH 7.4, 20 mM glutamine and 5 mM phosphate: ■, 0.5 mM spermine; ●, no added spermine.

Effects of NH_4^+ , phosphate, glutamine, pH and Mg^{2+}

(i) NH_4^+

Ammonia has been reported to be an obligatory activator of glutaminase under physiological conditions of phosphate and glutamine [9,25], although there is controversy as to whether the activating species is NH_3 [25] or NH_4^+ [9]. Since spermine contains four quaternary nitrogen atoms, it was important to determine its effect as a function of NH_4^+ concentration. As shown in Figure 4, spermine (0.5 mM) activated the enzyme at all NH_4^+ concentrations tested, and the effect of spermine was much greater than that of NH_4^+ . The NH_4Cl concentrations

Figure 5 Effect of phosphate concentration on glutaminase activity in the absence and presence of spermine

Glutaminase activity was assayed in freeze-thawed mitochondria, in the presence of 300 mM mannitol, 20 mM Hepes, pH 7.4, 20 mM glutamine and 0.7 mM NH_4Cl : ■, 0.5 mM spermine; ●, no added spermine.

shown on the abscissa of Figure 4 refer to added NH_4Cl . It is not possible to have a zero concentration of NH_4^+ , due to various ambient contaminations, and our estimate of this ammonia is about 0.05–0.1 mM. The spermine solution added did not contain any detectable ammonia. Although we cannot state that spermine activates in the complete absence of ammonia, it is clear from Figure 4 that spermine activates in the absence of added ammonia and that it is a much more potent activator than NH_4^+ or NH_3 . It is also clear that spermine greatly increases the sensitivity of glutaminase to activation by ammonia.

(ii) Phosphate

P_i is an obligatory activator of liver glutaminase. The half-maximal concentrations for P_i activation are pH-dependent. Szweda and Atkinson [10] report half-maximal activation by P_i at concentrations ranging from 9 mM at pH 7.7 to 21 mM at pH 7.1. In addition, the activity displays a sigmoidal dependence on P_i , indicative of co-operative binding of this ligand [11].

The data presented in Figure 5 show that spermine greatly increases the enzyme's affinity for P_i and, in addition, changes the curve of its dependence on P_i concentration from sigmoidal to hyperbolic. In fact the concentration of P_i required for half-maximal glutaminase activation in the presence of 0.5 mM spermine decreased to 4 mM from a value of 15 mM in its absence (results not shown).

(iii) Glutamine

Spermine greatly increased the affinity of glutaminase for its substrate (Figure 6). The dependence of velocity on glutamine concentration remains hyperbolic, but the apparent K_m values decrease from 38 mM to 15 mM in the presence of 0.5 mM spermine.

(iv) pH

The effect of pH on hepatic glutaminase is complex. The enzyme displays an alkaline pH optimum in most assays, but Szweda and

Figure 6 Dependence of glutaminase activity on glutamine concentration in the absence and presence of spermine

Glutaminase activity was assayed in freeze-thawed mitochondria, in the presence of 300 mM mannitol, 20 mM Hepes, pH 7.4, 5 mM phosphate and 0.7 mM NH_4Cl : ■, 0.5 mM spermine; ●, no added spermine.

Figure 7 Effect of H^+ concentration on glutaminase activity in the absence and presence of spermine

Glutaminase activity was assayed in freeze-thawed mitochondria, in the presence of 300 mM mannitol, 20 mM Hepes, at the pH shown, 20 mM glutamine, 5 mM phosphate and 0.7 mM NH_4Cl : ■, 0.5 mM spermine; ●, no added spermine.

Atkinson [10] have clearly shown that at saturating concentrations of the activators P_i and NH_4^+ (concentrations of 100 mM and 3.5 mM respectively) the activity at pH 7.1 was equal to that at 7.7. Since our interest was in the effect of spermine under conditions that approximate to those that may exist *in vivo*, we examined the effect of pH with lower concentrations of activators (5 mM P_i and 0.7 mM NH_4Cl). The data are given in Figure 7. In the absence of spermine, activity was low at pH 7.1 and 7.4, but was appreciably stimulated at pH 7.65. The presence of 0.5 mM spermine increased the activity at all pH values, but the effect was most pronounced at pH 7.4.

Figure 8 Effect of Mg^{2+} on glutaminase activity in the absence and presence of spermine

Glutaminase activity was assayed in freeze-thawed mitochondria in the presence of 300 mM mannitol, 20 mM Hepes, pH 7.4, 20 mM glutamine, 5 mM phosphate and 0.7 mM NH_4Cl : ■, 0.5 mM spermine; ▲, 0.1 mM spermine; ●, no added spermine.

(v) Mg^{2+}

Mg^{2+} ions have been reported to stimulate glutaminase [9]. Figure 8 shows that, in the absence of added spermine, glutaminase is stimulated by about 75% by 0.35 mM Mg^{2+} and by about 150% at 2 mM Mg^{2+} . Spermine (0.1 mM) stimulated glutaminase by 50% in the presence of 0.35 mM Mg^{2+} , the physiological matrix concentration [26,27]. At high Mg^{2+} concentration (2 mM), there was only a minor additional effect of spermine. At 0.5 mM spermine, there was no additional effect of Mg^{2+} .

DISCUSSION

Spermine has been shown to exert a number of effects in isolated mitochondria. In addition to its ability to increase mitochondrial Ca^{2+} accumulation [21], spermine has been reported to stabilize mitochondrial membranes and prevent mitochondrial swelling [28], and to prevent loss of respiratory control in ageing mitochondria and to restore this control after aging [29]. Spermine also maintains the mitochondrial membrane potential during Ca^{2+} and phosphate cycling and restores (in the presence of ATP) the membrane potential in de-energized mitochondria [30]. Many of these effects of spermine on mitochondrial integrity may be explained by the recent finding that spermine inhibits the permeability transition of isolated mitochondria [31]. Toninello et al. [32,33] have presented evidence for the electrophoretic transport of spermine into energized rat liver mitochondria. Spermine has been shown to activate the mitochondrial ATP synthase [34] and the ADP/ATP translocator [35].

The experiments reported in this paper clearly established spermine, at low concentration, to be a potent activator of hepatic glutaminase. In the presence of spermine, glutaminase becomes much more active under conditions that are more likely to represent those that exist *in vivo* in the mitochondrial matrix. Thus spermine makes the enzyme more sensitive to phosphate, increases its affinity for its substrate and makes it more active at pH 7.4. An important observation is that, in the presence of

spermine, added NH_3 or NH_4^+ is no longer required as an essential activator of hepatic glutaminase. However, it may be more significant that spermine greatly enhances the activation of glutaminase by ammonia.

In the present study, spermine and Mg^{2+} both increase glutaminase activity. At a physiological concentration of Mg^{2+} , however, spermine is still able to activate glutaminase. Similarly, pyruvate dehydrogenase phosphate phosphatase is activated by Mg^{2+} at a physiological concentration, and is further activated by spermine [36]. Although spermine is an organic cation, its geometry is quite different from that of Mg^{2+} or other inorganic cations, thus permitting distinctive interactions with macromolecules [37]. For example, spermine is an activator of the mitochondrial Ca^{2+} uniporter and Mg^{2+} is an antagonist [38].

For the effect of spermine on glutaminase to be of physiological significance, there must be sufficient free spermine in the mitochondrial matrix. The spermine content of rat liver mitochondria, isolated in a KCl medium to minimize adsorbed polyamines, was found to be 2.2 nmol/mg of mitochondrial protein (M. E. Brosnan, unpublished work). This amounts to a concentration of about 2 mM if all were free in the matrix space. Much of the spermine is likely to be bound to macromolecules, and free matrix concentrations are not known. However, there is more than enough total spermine in liver mitochondria to activate glutaminase.

This work was supported by grants from the Medical Research Council of Canada to J.T.B. and M.E.B. Z.K. was supported by an MRC of Canada East European Fellowship.

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