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Phosphate-dependent glutaminase in enterocyte mitochondria and its regulation by ammonium and other ions

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Summary. The effects of ammonium and other ions on phosphate dependent glutaminase (PDG) activity in intact rat enterocyte mitochondria were investigated. Sulphate and bicarbonate activated the enzyme in absence and presence of added phosphate. In presence of 10 mM phosphate, ammonium at concentrations <1 mM inhibited the enzyme. This inhibition was reversed by increased concentration of phosphate or sulphate. The inhibition of PDG by ammonium in presence of 10 mM phosphate was biphasic with respect to glutamine concentration, its effect being through a lowering of V_{max} at glutamine concentration of ≤5 mM, and increased K_m for substrate concentration above 5 mM. The activation of the enzyme by bicarbonate was through an increase in V_{max}. Ammonium and bicarbonate ions may therefore be important physiological regulators of PDG. It is suggested that phosphate and other polyvalent ions may function by preventing product inhibition of the enzyme through promotion of PDG dimer formation. The dimerized enzyme may have a high affinity for glutamine and reduced sensitivity to inhibition by ammonium ions.

Keywords: Glutaminase – Ammonium – Bicarbonate – Mitochondria – Enterocyte

Introduction

Glutamine is the most abundant amino acid in blood constituting 20% of the total amino acid content of plasma (Meister, 1980). The main source of this glutamine is skeletal muscle (Rudermann and Lund, 1972; Garber et al., 1976) with other issues including the brain (Lund, 1971) adipose tissue (Tischler and Goldberg, 1980; Frayn et al., 1991; Kowalski and Watford, 1994) and lungs (Welbourne, 1988) contributing. This amino acid plays a central role in nitrogen metabolism and is involved in purine, pyrimidine, NAD and glucosamine-6-phosphate synthesis as a nitrogen donor (Meister, 1980). It is a substrate for renal ammoniagenesis which is a mechanism for control of acidosis by the kidney (Van Slyke et al., 1943; Pitts et al., 1965; Squires et al., 1976). Glutamine is also a major respiratory fuel for rapidly dividing cells which include the intestinal mucosa (Windmueller and Spaeth, 1974; Windmeuller, 1982), lymphocytes (Ardawi and Newsholme, 1982; Newsholme et al., 1985) fibroblasts (Zeilke et al., 1976) and tumor cells (Kvamme and Svenneby, 1961; Reitzer et al., 1979). The absolute requirement of glutamine in energy provision in the intestinal mucosa has recently been questioned especially with respect to the ruminants, which show a different fate of glutamine taken up by portally drained viscera (Watford, 1999). Glutamine is also required by cells in culture (Zeilke et al., 1984). In injury or stress glutamine may be a conditionally essential amino acid due to its role in maintenance of gut metabolism, structure and function (Lacey and Wilmore, 1990; Souba et al., 1990; Elia, 1992).

Although there are a number of glutamine utilizing enzymes in the mucosa of the small intestine their activities are low in comparison to phosphate-dependent glutaminase (PDG) which is the dominant glutamine-utilizing enzyme in this tissue (Pinkus and Windmueller, 1977). The role of this enzyme in the metabolism of glutamine in the small intestine, its properties, and regulation by various factors was the subject of a recent review (McCauley et al., 1999). In common with other phosphate-dependent glutaminases, intestinal enzyme is localized in mitochondria (Pinkus and Windmueller, 1977). It has a differential distribution along the villus, crypt and lamina propria

axis its highest activity being found in the villus-crypt junction (Nagy et al., 1988; Shenoy et al., 1996). This region has, however, the lowest glutaminase protein concentration compared to the villus and crypt regions (Nagy et al., 1988). The small intestine accounts for 84% of the total glutaminase in the gastrointestinal tract which is in contrast to glutamine synthetase of which 32% is associated with the small intestine (James et al., 1998). In rats, intestinal PDG expression changes with maturation and increases in the 2nd and 3rd week of life (Shenoy et al., 1996; Nagy and Kretchmer, 1988). Glutaminase proteins of 72, 68 and 65 kDa are expressed in the small intestine. Similar glutaminase proteins are expressed in the kidney where the 72 KDa is the precursor (Shapiro et al., 1991). The expression of the intestinal PDG proteins varies according to maturity of animals. Differences in the expression of the protein are also seen between the proximal and distal parts of the small intestine (Shenoy et al., 1996) Additionally, glutaminase mRNA which has been shown to be localized in the villus and crypt epithelial cells, increases during the 3rd week of life (Shenoy et al., 1996).

A number of factors have been found to regulate PDG activity. Those that increase its activity include weaning (Hahn et al., 1998), late pregnancy and lactation (Ardawi, 1987), glucocorticoid administration to animals (Fox et al., 1988; Sarantos et al., 1992) or incubation with Caco-2 cells (Sarantos et al., 1994), and enteral branched chain amino acid enriched nutrient solutions (McCauley et al., 1997). Phosphate-dependent glutaminase activity is decreased in sepsis (Ardawi et al., 1990) and by cytokines interleukin and interferon-γ (Austgen et al., 1992; Sarantos et al., 1994). Studies on the effect of glutamine-enriched enteral and parenteral nutrition on glutaminase activity have been equivocal and it has been suggested that increases are only seen in catabolic animals (McCauley et al., 1999).

The short-term regulation of intestinal-phosphate dependent glutaminase has been little studied. The preliminary work of Pinkus and Windmueller (1977) has shown that the enzyme is activated to different degrees by 67 mM concentrations of various compounds which include phosphate, ammonium sulphate, glucose-6-phosphate, sulphate, maleate, ammonium bicarbonate, citric, pyrophosphate and oxalate. In this paper we examine the effects of some of these compounds at lower concentrations in intact freshly prepared mitochondria. We also investigate the effects of ammonium ions on the activity of the enzyme.

Materials and methods

Animals

Male Sprague-Dawley rats bred at the University of Zimbabwe Animal House were used in all experiments. The rats, which were maintained on a diet of rat chow pellets and allowed free access to water, weighed 250–300 g.

Chemicals

Perchloric acid (60%, AR) was obtained from Riedel-deHaën, Germany. Bovine serum albumin, sucrose, ADP di-sodium salt, β-NAD^+ sodium salt, L-glutamine, L-glutamate, L-malate, mannitol, EGTA, HEPES, Tris-HCl, glycine, hydrazine, L-glutamate dehydrogenase in 50% glycerol, $KHCO_3$, NaCl, $NaHCO_3$, KCl, KH_2PO_4, $MgSO_4{\cdot}6H_2O$, K_2SO_4, NH_4Cl, $(NH_4)_2SO_4$ and aminooxyacetate were obtained from Sigma Chemical Company, South Africa. Double distilled water was used for making solutions. Solutions of L-glutamate, ADP, NAD and $KHCO_3$ were prepared freshly.

Preparation of enterocytes and enterocyte mitochondria

Enterocytes were prepared from two rats according to the method of Watford et al. (1979) modified as described by Masola and Evered (1984). Mitochondria were prepared freshly as described by Masola and Evered (1984), however the fraction of unbroken cells and nuclei was re-homogenized with five passes of the Potter Evelhejm homogenizer to increase mitochondria yield. The preparation medium consisted of 250 mM sucrose, 5 mM HEPES, 1 mM EGTA, pH 7.4.

Mitochondrial respiratory experiments

Mitochondrial respiration was measured at 30°C using a Rank Oxygen Electrode (Rank Brothers, Bottisham, England). The incubation medium consisted of 100 mM KCl, 25 mM sucrose, 75 mM mannitol, 10 mM KH_2PO_4, 10 mM HEPES, 1 mM EGTA and 0.1% w/v fatty acid free bovine serum albumin. The respiratory control and P/O ratios were determined as described by Chappell (1964) and Estabrook (1967).

Glutaminase assay

Phosphate dependent glutaminase was assayed at 37°C in a two step procedure essentially as described by Curthoys and Weiss (1974) but with modifications. The initial reaction mixture consisted of 200 μl of 120 mM KCl, 5 mM aminooxyacetate, 5 mM KCN, 50 mM Tris-HCl and varying concentrations of glutamine and phosphate, pH 7.4. Reactions were started by addition of 50 μl enzyme diluted when appropriate, and terminated after 15 minutes by addition of cold perchloric acid to a final concentration of 10%. After allowing the mixture to stand in ice for 5 minutes it was neutralized with KOH and centrifuged. The glutamate formed was determined in the supernatant as described by Bernt and Bergmeyer (1974).

Statistical analysis

Graphs were plotted using Graph Pad Prism programme, version 3.0 whereas means were compared by the t-test using Graph Pad Instat programme, version 3.0 (Graph Pad Software Inc., USA).

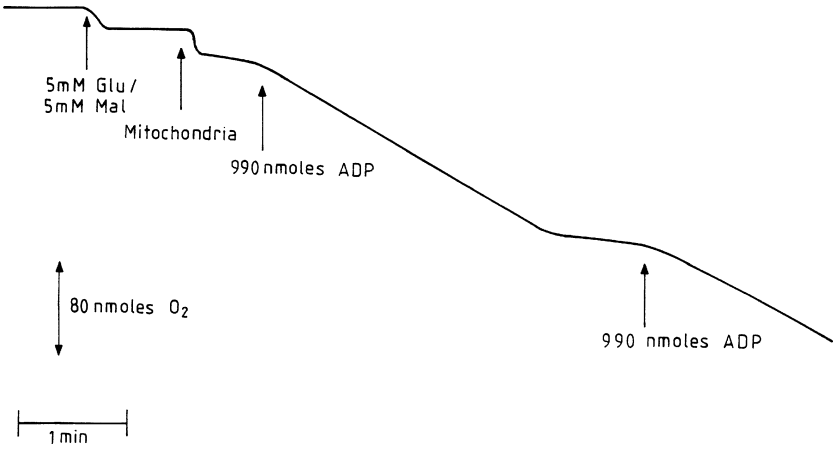

Fig. 1. Oxygen electrode trace of respiring rat enterocyte mitochondria at 30°C. The incubation medium consisted of 120 mM KCl, 25 mM Sucrose, 75 mM mannitol, 10 mM KH_2PO_4, 10 mM HEPES, 1 mM EGTA and 0.1% w/v fatty acid free bovine serum albumin, pH 7.4. Approximately 3 mg of mitochondria were added in a final incubation volume of about 3.2 ml

Results

Mitochondrial respiration

The results in Fig. 1. show an oxygen electrode trace of respiring enterocyte mitochondria. The respiratory control ratio was 5 whereas the P : O ratio was 3, showing functional integrity of mitochondria used in these experiments. The data in each of Figs. 2–6 were generated from several mitochondrial preparations.

The effects of different ions on glutaminase activity in absence and presence of 10 mM phosphate with 20 mM glutamine as the substrate

The effects of various ions on glutaminase activity in absence and presence of 10 mM phosphate are shown in Figs. 2 and 3. Since potassium and chloride ions were present in excess the effects observed are assumed to be due to ammonium, bicarbonate, sulphate and phosphate ions. In presence of 10 mM phosphate the initial activity of PDG determined in absence of other added ions was higher than in its absence showing the activating effect of phosphate. The level of activation of the enzyme due to added ions was however higher in absence of phosphate with 10 mM concentrations of K_2SO_4, $KHCO_3$ and $(NH_4)_2SO_4$, respectively, increasing the initial activity of the enzyme by approximately 4.7, 3 and 3-fold compared to 1.6, 1.4 and 0-fold in presence of 10 mM phosphate. There was no activation of the enzyme due to NH_4Cl. The results in Fig. 3 in fact show that 1 mM concentrations of both ammonium salts inhibited the enzyme by 45%. Increasing concentrations of $(NH_4)SO_4$ overcame this inhibition but this was not the case with NH_4Cl, showing that higher concentrations of the sulphate ions were responsible for overcoming inhibition due to the ammonium ions.

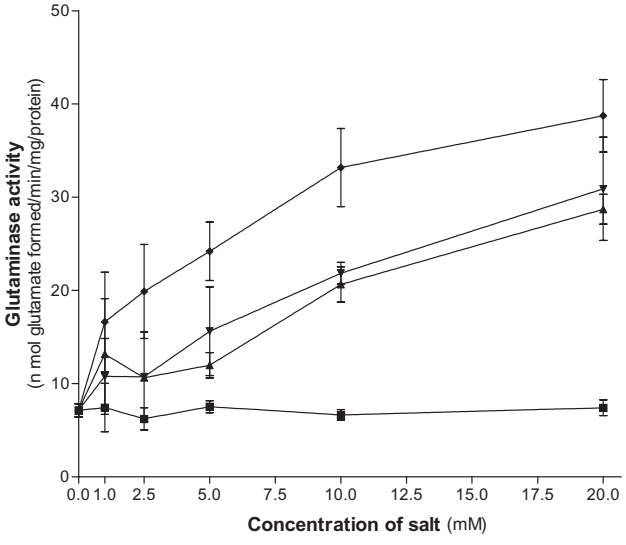

Fig. 2. Effects of varying concentrations of K_2SO_4 (-◆-), $KHCO_3$ (-▼-), $(NH4)_2SO_4$ (-▲-) and NH_4Cl (-■-) on glutaminase activity in intact rat enterocyte mitochondria, in the absence of added phosphate. The initial concentration of the substrate glutamine was 20 mM. Results are means ± SEM for n = 4 duplicate determinations

At a concentration of 20 mM $KHCO_3$, the activity of glutaminase was increased respectively by 4.4 and 1.8-fold in absence and presence of 10 mM phosphate when compared to initial activities in absence of added bicarbonate.

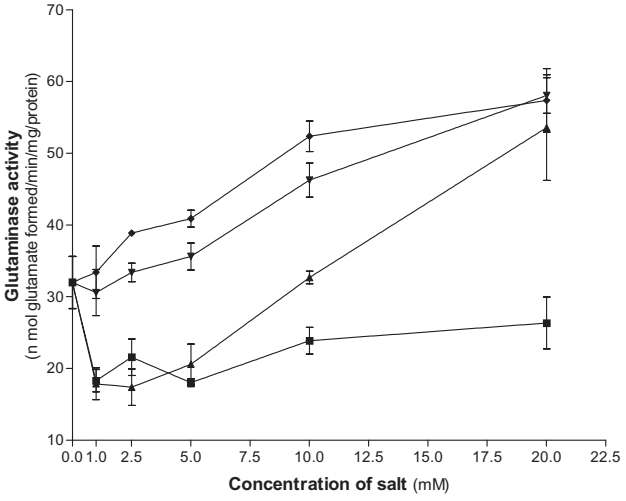

Fig. 3. Effects of varying concentrations of K_2SO_4 (-◆-) $KHCO_3$ (-▼-), $(NH_4)_2SO_4$ (-▲-) and NH_4Cl (-■-) on glutaminase activity in intact rat enterocyte mitochondria, in presence of 10 mM phosphate. The initial concentration of the substrate glutamine was 20 mM. Results are means ± SEM for n = 3–4 duplicate determinations

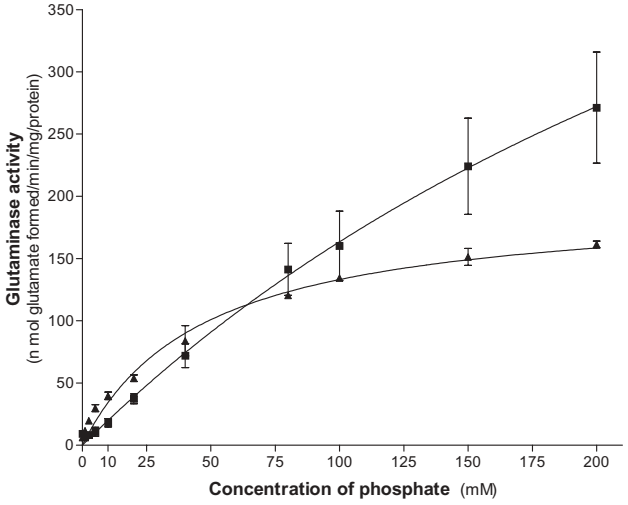

Fig. 4. Effects of varying the concentration of phosphate on glutaminase activity in intact rat enterocyte mitochondria in presence (-■-) and absence (-▲-) of 1 mM ammonium chloride. The initial concentration of the substrate glutamine was 20 mM. Results are means ± SEM for n = 3 duplicate determinations

Effect of ammonium ions on glutaminase activity in relation to phosphate concentration with 20 mM glutamine as the substrate

The results in Fig. 3 show that there was a significant inhibitory effect of 1 mM NH_4Cl on PDG activity in presence of 10 mM phosphate, however, at the high phosphate concentration of 150 mM no such inhibitory effect was observed (results not shown). The results in Fig. 4 show the effect of 1 mM NH_4Cl on glutaminase activity at different concentrations of phosphate. Statistical comparison of PDG activities in presence and absence of NH_4Cl showed that there were only significant differences ($P < 0.05$) at concentrations of phosphate of up to 20 mM, the exception being at zero phosphate concentration. There was, however, no significant enzyme activity difference at concentrations of phosphate of 40 mM and above in presence and absence of 1 mM NH_4Cl.

Changes in kinetic constants for glutaminase with respect to glutamine in absence and presence of added ammonium and bicarbonate ions

Results in Fig. 5a show the variation of enzyme activity with increasing concentration of glutamine in absence and presence of ammonium chloride. The results were re-plotted in Fig. 5b using the Hanes plot in which $[S_o]/V_o$ is plotted against $[S_o]$ where $[S_o]$ is the substrate concentration and V_o the initial velocity obtained at each substrate concentration. V_{max}, the maximum velocity is then given by 1/slope whereas K_m is given by V_{max} x intercept on the $[S_o]/V_o$ axis. Similarly the variation of enzyme activity with increasing concentration of substrate in absence and presence of potassium bicarbonate is shown in Fig. 6a and in Fig. 6b where the results are re-plotted using the Hanes plot.

The kinetic constants determined using the plots above are shown in Table 1. The values for K_m and V_{max} in absence of added salts are averages of the two values obtained for each constant in Figs. 5b and 6b. The effect of 1 mM NH_4Cl on glutaminase activity was biphasic with respect to glutamine concentrations shown in Fig. 5b where there was a break in the linearity of the Hanes plot at 5 mM glutamine concentration. At low (≤5 mM) glutamine concentration, K_m was lowered 42% and V_{max} by 57% with only the decrease in V_{max} being significant ($P < 0.05$). At high (>5 mM) glutamine concentration, K_m was increased by 520% and V_{max} by 9% with only the increase in K_m being significant.

The effect of 20 mM $KHCO_3$ on glutaminase activity is shown in Fig. 6b, the changes in K_m and V_{max} being highlighted in Table 1. K_m for glutamine decreased by 17% whereas V_{max} increased by 72% due to the presence of bicarbonate. Only the increase in V_{max} was significant ($P < 0.05$).

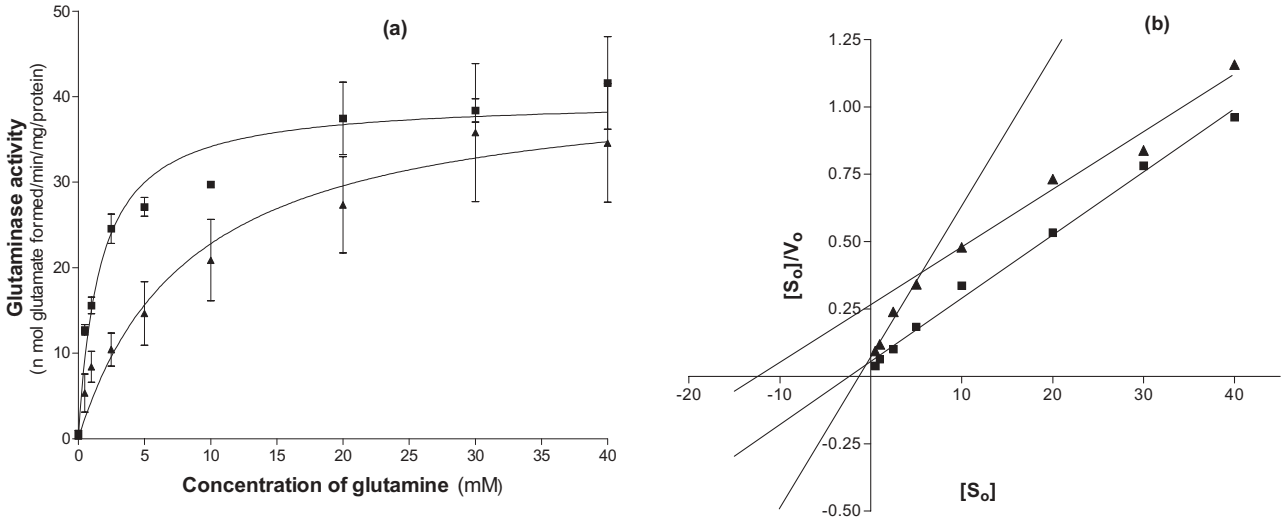

Fig. 5. **a** Effects of varying the concentration of glutamine on glutaminase activity in intact mitochondria in absence (-■-) and presence (-▲-) of 1 mM ammonium chloride. **b** The results in (a) re-plotted using the Hanes plot. The concentration of phosphate in the assays was 10 mM. Results are means ± SEM for n = 3 duplicate determinations

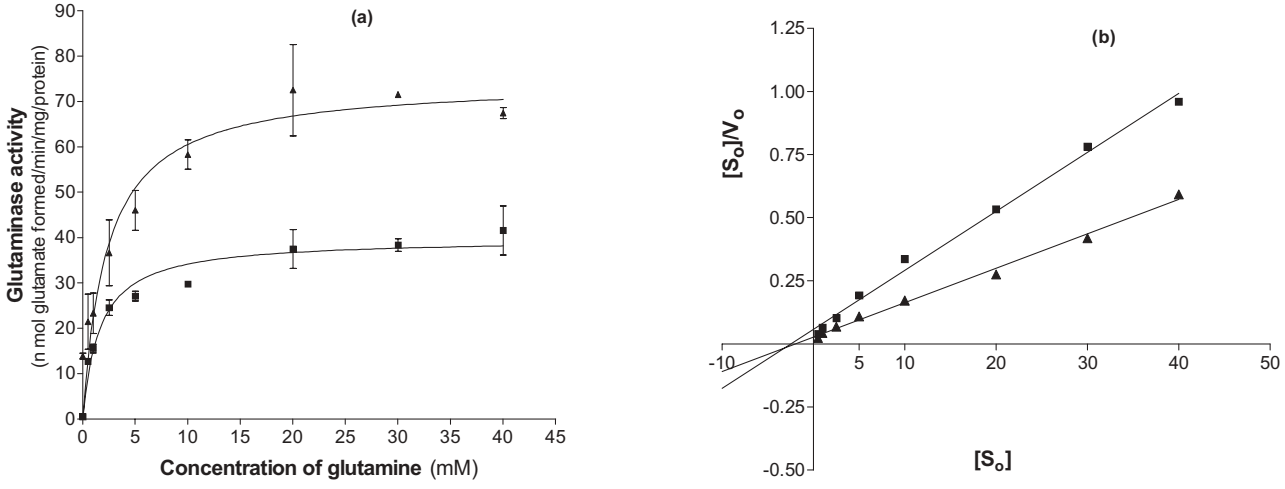

Fig. 6. **a** Effects of varying the concentration of glutamine on glutaminase activity in intact mitochondria in absence (-■-) and presence (-▲-) of 20 mM potassium bicarbonate. **b** The results in (a) re-plotted using the Hanes plot. The concentration of phosphate in the assays was 10 mM. Results are means ± SEM for n = 3 duplicate determinations

Table 1. Kinetic constants for glutaminase with respect to glutamine in absence and presence of ammonium and bicarbonate ions

Kinetic constant	No NH_4Cl or $KHCO_3$ present	1 mM NH_4Cl	20 mM $KHCO_3$
K_m (mM)	2.38	1.37 ([S] <5 mM) 12.4* ([S] >5 mM)	1.98
V_{max} (n moles Glutamate formed/ min/mg protein)	41.6	18.3* ([S] <5 mM) 46.7 ([S] >5 mM)	73.4*

[S], glutamine concentration, * Result in presence of NH_4Cl or $KHCO_3$ was significantly different ($P < 0.05$) from that in their absence. Assay conditions were as described in the methods section and in legends of Figs. 5 and 6

Discussion

The results of the current study using respiring mitochondria show that phosphate, sulphate and bicarbonate ions are potent activators of phosphate dependent glutaminase. The activation of intestinal phosphate dependent glutaminase by sulphate and other polyvalent ions was also demonstrated by Pinkus and Windmueller (1977) although concentrations used were high. Rat kidney enzyme is similarly activated by polyvalent ions including nucleotides (Curthoys et al., 1976). The activation of the kidney enzyme by phosphate is due to promotion of dimerization that results in the active form of the enzyme. The dimer has a low K_m for glutamine and a high K_m for glutamate whereas the inactive protomeric form has a high K_m for glutamine and a low K_m for glutamate (Curthoys et al., 1984). The similarity in structures of phosphate and sulphate suggests that the latter may be activating the enzyme in a manner similar to that of phosphate.

The current results also show that intestinal PDG is strongly activated by bicarbonate both in absence and presence of added phosphate. This activation is through an increase in V_{max} rather than substrate binding affinity. Baverel and Lund (1979) observed that bicarbonate increased glutamine removal by isolated enterocytes and hepatocytes but not by kidney cortex tubules in which it inhibited glutamate dehydrogenase. Bicarbonate has also been shown to activate PDG in isolated rat liver mitochondria (Joseph and McGivan, 1978). The activation of intestinal PDG by bicarbonate could be important physiologically since the ion is released in large quantities from the pancreas to neutralize acid contents of the stomach as they enter the duodenum and subsequently the small intestine. The resulting alkaline conditions and high concentration of bicarbonate would thus stimulate PDG and hence increase energy generation for transport and other cellular activities.

Intestinal phosphate dependent glutaminase is of "kidney" type in common with PDGs from brain, foetal liver and Ehrlich tumour cells which are different from the "liver type" in terms of polymerization in borate buffer, mRNAs, subunit composition and immunological properties (Watford, 1993). Intestinal PDG appears to have similar kinetic properties to kidney PDG. They have similar K_ms for glutamine and phosphate of 2–5 mM and 20–30 mM respectively (Pinkus and Windmueller, 1977; Curthoys et al., 1976; present results). The pig kidney cortex enzyme has been shown to be activated by ammonium ions at concentrations above 25 mM (Kvamme et al., 1970), however our results (unpublished) show that at the high phosphate concentration 150 mM, there was no significant change in intestinal PDG activity for a range of NH_4Cl concentrations of up to 20 mM. The enzyme was, however, inhibited by NH_4Cl in presence of low (10 mM) concentration of phosphate (Figs. 3 and 4). In fact NH_4Cl had a significant inhibitory effect on PDG at concentrations of phosphate below 40 mM but not above (Fig. 4), which may indicate physiological significance of this phenomenon. It may also be noted that liver mitochondria can accumulate phosphate to achieve intramitochondrial concentrations of approximately 9 mM in presence of low external concentrations of phosphate (Lacey et al., 1981). This may represent the physiological range of intramitochondrial phosphate concentrations. Our results further show that the inhibitory effects of NH_4Cl are dependent on glutamine concentration. At low glutamine concentration the effects are through lowering of V_{max} which indicates interference with breakdown of enzyme – substrate complexes to products. Since ammonia is a product of the glutaminase reaction, it may thus have serious effects on intestinal glutaminase activity if its removal via the portal vein is impaired. At high glutamine concentrations (>5 mM) which are in the non-physiological range, the inhibitory effect of NH_4Cl was through a decrease in the binding affinity for substrate with V_{max} largely unaffected indicating possible competitive inhibition. The response of intestinal PDG is somewhat similar to that of rat brain synaptosomal PDG with respect to the inhibitory effect of ammonium ions in presence of low concentrations of phosphate (Kvamme, 1984; Wallace and Dawson, 1992). The pancreatic islets enzyme is, however, not inhibited by low concentrations of ammonium ions in presence of low phosphate concentration (Michalik et al., 1992).

Since phosphate has been shown to promote dimerization of kidney PDG (Curthoys et al., 1984) we hypothesize that the intestinal enzyme responds similarly. The presence of phosphate (or other polyvalent ions e.g. sulphate) would therefore result in an increasingly dimerized form of the enzyme which has a high affinity for glutamine. It is interesting to note that the initial inhibitory effect of ammonium sulphate is reversed at higher concentrations of the salt (Fig. 3). It would appear, therefore, that increased dimerization

of the enzyme due to higher concentrations polyvalent ions like sulphate prevents inhibition of the enzyme by its product ammonia. This may be physiologically important as the intestine and other visceral organs generate large quantities of ammonium whose concentration is in the range 0.5–0.8 mM in portal vein blood (Watford, 1993). The overall effect of dimerization in the physiological range of glutamine concentrations would be to increase the affinity of intestinal PDG for glutamine and to prevent the inhibitory effect of ammonium ions that lower V_{max}.

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