Inhibition of Glutamine Synthetase Activity by Manganous Ions in a Cytosol Extract of Rat Liver

By Suresh K. JOSEPH,* Norah M. BRADFORD and John D. MCGIVAN
Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

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Glutamine synthetase activity in a cytosol extract of liver was inhibited non-competitively by Mn^{2+} ions. The apparent K_i for Mn^{2+} in the presence of phosphate was $8\mu M$. Inhibition of glutamine synthetase by intracellular Mn^{2+} may contribute to the very low rates of glutamine synthesis observed in perfused liver and isolated hepatocytes.

The major tissue responsible for the net synthesis of glutamine in mammals is skeletal muscle. Whether or not the liver also contributes significantly to the net synthesis of glutamine is unclear. The evidence obtained from experiments measuring arteriovenous differences in the plasma concentration of this amino acid across the liver *in vivo* is contradictory and has been reviewed by Lund & Watford (1976). In metabolic acidosis, an increased uptake of glutamine by the kidney is observed. It has been proposed that the hepatic synthesis of glutamine may be of importance in the supply of this amino acid to the kidney under these conditions (Lotspeich, 1967).

The rate of glutamine synthesis in perfused liver has been found to represent only one-fortieth of the catalytic activity of glutamine synthetase (EC 6.3.1.2) as assayed in crude extracts of rat liver (Lund, 1971), which led to the suggestion that this enzyme may be inhibited in the liver cell, but the factors responsible for such an inhibition have not been identified. Although purified glutamine synthetase from rat liver is inhibited by carbamoyl phosphate, alanine, glycine and phosphate, these inhibitory effects are large only when Mn^{2+} replaces Mg^{2+} in the assay medium (Tate & Meister, 1971), and the significance of these effects in the physiological regulation of glutamine synthetase is doubtful. The present investigation was undertaken in order to identify inhibitors of glutamine synthetase that may be responsible for the apparent low activity of this enzyme in the intact cell.

Experimental

A cytosol extract of rat liver was prepared essentially as described by Moss & McGivan (1975). The liver from a normally fed 200g female Wistar rat was homogenized in 40ml of a medium containing 100mM-KCl, 20mM-Tris/HCl and 20mM-MgCl₂

* Present address: Laboratory of Biochemistry, B. C. P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, 1018TV, Amsterdam, The Netherlands.

at pH 7.4 and 4°C. The homogenate was centrifuged at 1000g for 10min, and the supernatant was then centrifuged at 38000g for 30min to ensure sedimentation of subcellular organelles. The supernatant from the final centrifugation was depleted of endogenous substrates by passing 5ml portions down a column (2cm×15cm) of Sephadex G-25 (coarse grade) in the cold. The final solution contained 10–15mg of protein/ml and had a glutamine synthetase activity of approx. 40nmol/min per mg at 37°C.

Glutamine synthetase activity in this extract was routinely determined by assaying the complex formed between glutamylhydroxamate and Fe^{3+} ions, with hydroxylamine as a substrate in place of NH_3 (Wellner & Meister, 1966). Unless otherwise stated, the incubation medium contained (final concentrations): 80mM-KCl, 10mM-Mops (4-morpholinepropanesulphonate; potassium salt), 2mM-ATP, 10mM-MgCl₂, 20mM-hydroxylamine hydrochloride, 10mM-glutamate, 2mM-phosphocreatine, 0.1mg of creatine kinase (Boehringer)/ml and 3–4mg of protein/ml at pH 7.4 and 37°C. When 20mM-KHCO₃ was also present, the medium was equilibrated with O₂/CO₂ (19:1). The reaction was terminated by addition of an FeCl₃-containing deproteinizing solution (Wellner & Meister, 1966). Preliminary experiments showed that the time course of glutamylhydroxamate formation was linear over the period employed. The use of an ATP-regenerating system was necessary, since the cytosol extract contains considerable activities of ATPase and adenylate kinase (Moss & McGivan, 1975). The pH of the medium remained constant throughout the incubation.

In some experiments, glutamine synthetase activity was measured with NH_3 as substrate. In one method, the formation of [¹⁴C]glutamine from [¹⁴C]glutamate was measured by separation of these compounds on Dowex ion-exchange columns. In another method, the cytosol extract was incubated with appropriate concentrations of glutamate, NH_4 Cl, MgCl₂ and ATP. The formation of ADP by the glutamine synthetase reaction was assayed con-

tinuously by coupling ADP production to the oxidation of NADH by the addition of phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase in excess to the same cuvette. NADH oxidation was measured at 340nm in a split-beam spectrophotometer. The control cuvette contained all the reactants except NH_4Cl , and hence any contribution by endogenous ATPase to the rate was eliminated. Essentially similar results were obtained for glutamine synthetase activity by all three assay methods.

Results

Fig. 1 shows the effect of the addition of various bivalent cations on the activity of glutamine synthetase in a cytosol extract in the presence of a saturating concentration of Mg^{2+} . It was found that Ca^{2+} , Mn^{2+} and Zn^{2+} inhibited the enzyme. Mn^{2+} was the most effective of these inhibitors. Purified glutamine synthetase from rat liver has been shown to be inhibited by Mn^{2+} , even in the presence of a saturating concentration of Mg^{2+} (Tate & Meister, 1971). In this case, the concentration of Mn^{2+} required to cause 50% inhibition was $150\mu\text{M}$. However, the enzyme assayed in a crude extract of rat liver appears to be more sensitive to inhibition

Fig. 1. Inhibition of glutamine synthetase activity by bivalent cations

The incubation conditions were those described in the Experimental section and the incubation time was 10min. Additions at the concentrations shown were as follows: Δ , Ca^{2+} ; $\circ$, Zn^{2+} ; $\bullet$, Mn^{2+} . Glutamine synthetase activity is expressed as nmol of glutamylhydroxamate synthesized/min per mg of cytosol protein.

by Mn^{2+} ; in the experiment in Fig. 1, 50% inhibition was obtained at $20\mu\text{M-Mn}^{2+}$. The concentration of Mn^{2+} required for 50% inhibition of glutamine synthetase activity in this system showed a slight dependence on the concentration of Mg^{2+} present; 50% inhibition was obtained at $30\mu\text{M-Mn}^{2+}$ when the Mg^{2+} concentration was increased to 100mM .

The effects of certain anions on glutamine synthetase activity are shown in Fig. 2. As described previously for the rat liver enzyme, carbamoyl phosphate and phosphate are inhibitory (Tate & Meister, 1971; Tate *et al.*, 1972). Bicarbonate, a potent activator of glutaminase in liver mitochondria (Joseph & McGivan, 1978), was found to have a small inhibitory effect on glutamine synthetase activity under the conditions used.

The factor responsible for inhibiting glutamine synthetase in the intact cell would be expected to decrease the activity of the enzyme by more than 90% in the presence of excess substrate concentrations, and would therefore be non-competitive with all the substrates of this enzyme. Fig. 2 shows that maximal inhibition by bicarbonate, phosphate and carbamoyl phosphate was less than 50%. Further, experiments in which the activity of the enzyme was measured at various ATP concentrations in the presence of an ATP-regenerating system showed that the inhibitory effect of bicarbonate was com-

Fig. 2. Inhibition of glutamine synthetase activity by bicarbonate, phosphate and carbamoyl phosphate. Conditions were as for Fig. 1. Additions at the concentrations shown were: $\bullet$, KHCO_3 ; Δ , potassium phosphate; $\square$, potassium carbamoyl phosphate.

Table 1. Apparent K_i values for the inhibition of glutamine synthetase activity by Mn^{2+}

The values shown are taken from a series of experiments of the type shown in Fig. 1 performed over a period of 3 months. In these experiments, the effect of different concentrations of Mn^{2+} on the activity of glutamine synthetase was observed at various concentrations of each of the substrates at protein concentrations between 1.5 and 3.5 mg/ml. For each observation, the apparent K_i was calculated from the following relationship for a non-competitive inhibitor (Hunter & Downs, 1945):

$$K_i = i \left(\frac{x}{1-x} \right)$$

where i is the inhibitor concentration and x is the ratio (rate in the presence of Mn^{2+})/(rate in the absence of Mn^{2+}). The results are presented as means $\pm$ S.E.M. for the numbers of observations shown in parentheses. Levels of significance are represented as: * $P < 0.001$ versus control; † $P < 0.001$ versus K_i in the presence of bicarbonate. The results in lines 1 and 2 were obtained from 12 different preparations of cytosol extract.

Addition to standard incubation medium	Apparent K_i for Mn^{2+} (μM)
None	20.16 $\pm$ 0.66 (40)
KHCO ₃ (20 mM)	11.59 $\pm$ 0.57 (20)*
Potassium phosphate (5 mM)	7.93 $\pm$ 0.42 (18)*†
Potassium carbamoyl phosphate (5 mM)	10.90 $\pm$ 0.51 (3)*

petitive with respect to ATP. The inhibition of purified glutamine synthetase by phosphate is also competitive with ATP (Tate *et al.*, 1972). The concentration of carbamoyl phosphate required to produce an inhibitory effect on the enzyme is much higher than the concentration at which this compound is likely to be present in the cytosol.

Of the inhibitors tested, only Mn^{2+} fulfils the requirements for an inhibitor that is likely to be effective at physiological concentrations. In a series of experiments, it was determined that Mn^{2+} is a non-competitive inhibitor with respect to ATP, glutamate and hydroxylamine. The apparent K_i for inhibition by Mn^{2+} depended on the presence or absence of phosphate, carbamoyl phosphate or bicarbonate. These anions appeared to potentiate the inhibitory effects of limiting concentrations of Mn^{2+} . Table 1 shows the results of a large number of experiments in which glutamine synthetase activity was determined in the presence of various concentrations of ATP, glutamate, hydroxylamine, Mn^{2+} and protein in the presence and absence of 20 mM-KHCO₃, 5 mM-potassium phosphate or 5 mM-carbamoyl phosphate. The apparent K_i value for the inhibition of glutamine synthetase by Mn^{2+} was markedly decreased in the presence of these compounds.

Control experiments (results not shown) established that the kinetics of inhibition by Mn^{2+} did not change when the ATP-regenerating system was omitted from the assay medium and replaced by higher concentrations of ATP. Further, the K_i for inhibition of the enzyme by Mn^{2+} was unchanged when the enzyme was assayed with NH₄Cl as substrate by either of the methods described above.

Discussion

In the present study, a cytosol extract of rat liver has been used to evaluate potential physiologically important inhibitors of glutamine synthetase. The results showed that Mn^{2+} inhibited the enzyme, and that in the presence of physiological concentrations of phosphate or bicarbonate the apparent K_i for Mn^{2+} was 8–10 μM . The concentration of free Mn^{2+} ions under the conditions used in the present investigation is unknown, since the degree to which Mn^{2+} binds to proteins and ions such as ATP, glutamate and phosphate in the presence of 10 mM-Mg²⁺ is not easy to estimate. Purified Mg²⁺-activated rat liver glutamine synthetase is inhibited by Mn^{2+} only at much higher concentrations (Tate & Meister, 1971). It is possible that during the purification process some of the sensitivity to Mn^{2+} ions is lost. The finding that HCO₃⁻ and phosphate ions apparently make the enzyme more sensitive to Mn^{2+} cannot be interpreted in mechanistic terms from the present data.

It has been noted previously that the rate of synthesis of glutamine in perfused liver or isolated hepatocytes is very much less than the full capacity of glutamine synthetase in liver extracts. The K_m values of the purified enzyme for all its substrates are considerably lower than the concentrations of these substrates that are likely to occur in the liver cytoplasm. Thus the activity of the enzyme is unlikely to be limited by substrate availability *in vivo* (Lund & Watford, 1976; Lund, 1971). The results of the present work suggest that a major factor determining the low activity of glutamine synthetase in the intact cell may be inhibition by cytoplasmic Mn^{2+} . The total Mn^{2+} content of perfused liver has been reported to be 50–60 nmol/g wet wt. (Wimhurst & Manchester, 1973). If only 10% of this were free in the cytoplasm, this would be sufficient to cause a severe inhibition of glutamine synthetase.

It follows from the present study that, at a fixed concentration of Mn^{2+} , the activity of glutamine synthetase can be varied by no more than 2-fold by varying the concentration of bicarbonate or of phosphate in the physiological range. However, very much larger increases in activity could be obtained as a result of decreasing the cytoplasmic Mn^{2+} concentration. Whether such changes in Mn^{2+} con-

centration in the liver cytoplasm occur under any metabolic conditions is unknown at present.

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