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

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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 nM. 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 200 g female Wistar rat was homogenized in 40 ml of a medium containing 100 mM-KCl, 20 mM-Tris/HCl and 20 mM-MgCl$_2$ at pH 7.4 and 4°C. The homogenate was centrifuged at 1000 g for 10 min, and the supernatant was then centrifuged at 38 000 g for 30 min to sediment subcellular organelles. The supernatant from the final centrifugation was depleted of endogenous substrates by passing 5 ml portions down a column (2 cm x 15 cm) of Sephadex G-25 (coarse grade) in the cold. The final solution contained 10-15 mg of protein/ml and had a glutamine synthetase activity of approx. 40 nmol/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): 80 mM-KCl, 10 mM-Mops (4-morpholinepropanesulphonate; potassium salt), 2 mM-ATP, 10 mM-MgCl$_2$, 20 mM-hydroxylamine hydrochloride, 10 mM-glutamate, 2 mM-phosphocreatine, 0.1 mM of creatine kinase (Boehringer)/ml and 3-4 mg of protein/ml at pH 7.4 and 37°C. When 20 mM-KHCO$_3$ was also present, the medium was equilibrated with O$_2$/CO$_2$ (19:1). The reaction was terminated by addition of an FeCl$_3$-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 adenylyl 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 $[^14]$Cglutamine from $[^14]$Cglutamate 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, MgCl$_2$ and ATP. The formation of ADP by the glutamine synthetase reaction was assayed con-
continuously 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 340 nm in a split-beam spectrophotometer. The control cuvette contained all the reactants except \( \text{NH}_4\text{Cl} \), 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 \( \text{Mg}^{2+} \). It was found that \( \text{Ca}^{2+} \), \( \text{Mn}^{2+} \) and \( \text{Zn}^{2+} \) inhibited the enzyme. \( \text{Mn}^{2+} \) was the most effective of these inhibitors. Purified glutamine synthetase from rat liver has been shown to be inhibited by \( \text{Mn}^{2+} \), even in the presence of a saturating concentration of \( \text{Mg}^{2+} \) (Tate & Meister, 1971). In this case, the concentration of \( \text{Mn}^{2+} \) 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 by \( \text{Mn}^{2+} \); in the experiment in Fig. 1, 50% inhibition was obtained at 20 \( \mu \text{M} \)-\( \text{Mn}^{2+} \). The concentration of \( \text{Mn}^{2+} \) required for 50% inhibition of glutamine synthetase activity in this system showed a slight dependence on the concentration of \( \text{Mg}^{2+} \) present; 50% inhibition was obtained at 30 \( \mu \text{M} \)-\( \text{Mg}^{2+} \) when the \( \text{Mg}^{2+} \) concentration was increased to 100 mm.

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-
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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 differential 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 ± S.E.M. for the number 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.

<table>
<thead>
<tr>
<th>Addition to standard incubation medium</th>
<th>Apparent $K_i$ for Mn$^{2+}$ (μM)</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>20.16 ± 0.66 (40)</td>
</tr>
<tr>
<td>KHCO$_3$ (20 mm)</td>
<td>11.59 ± 0.57 (20)*</td>
</tr>
<tr>
<td>Potassium phosphate (5 mm)</td>
<td>7.93 ± 0.42 (18)*†</td>
</tr>
<tr>
<td>Potassium carbamoyl phosphate (5 mm)</td>
<td>10.90 ± 0.51 (3)*</td>
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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 NH$_4$Cl 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-Mg$^{2+}$ is not easy to estimate. Purified Mg$^{2+}$-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 HCO$_3^-$ 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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References