Plant Physiol. (1975) 55, 968-974

Pea Leaf Glutamine Synthetase

REGULATORY PROPERTIES[1]

Received for publication October 30, 1974 and in revised form February 4, 1975

THOMAS DENNY O'NEAL AND KENNETH W. JOY[2]
Department of Biology, Rensselaer Polytechnic Institute, Troy, New York 12181

ABSTRACT

Of a variety of purine and pyrimidine nucleotides tested, only ADP and 5'AMP significantly inhibited the Mg^{2+}-dependent activity of pea leaf glutamine synthetase. They were less effective inhibitors where Mn^{2+} replaced Mg^{2+}. They were competitive inhibitors with respect to ATP, with inhibition constant (Ki) values of 1.2 and 1.8 mM, respectively. The energy charge significantly affects the activity of glutamine synthetase, especially with Mg^{2+}. Of a variety of amino acids tested, L-histidine and L-ornithine were the most inhibitory, but significant inhibition was seen only where Mn^{2+} was present. Both amino acids appeared to compete with L-glutamate, and the Ki values were 1.9 mM for L-histidine (pH 6.2) and 7.8 mM for L-ornithine (pH 6.2). L-Alanine, glycine, and L-serine caused slight inhibition (Mn^{2+}-dependent activity) and were not competitive with ATP or L-glutamate.

Carbamyl phosphate was an effective inhibitor only when Mn^{2+} was present, and did not compete with substrates. Inorganic phosphate and pyrophosphate caused significant inhibition of the Mg^{2+}-dependent activity.

Since glutamine synthetase (EC 6.3.1.2) catalyzes the first reaction in the complex branched pathways leading to the synthesis of a number of basic products, such as tryptophan, histidine, CAP[3], purine nucleotides, pyridine nucleotides, and glycine, serine, and alanine (5, 6), it was considered likely that it would prove to be a regulatory enzyme, as has been shown to be the case for many other enzymes occupying similar positions in other branched pathways (20).

Glutamine synthetase of a number of microorganisms is subject to feedback by a variety of end products, although there is considerable variation between species or genera with regard to the most effective compounds. When two or more inhibitors were added simultaneously, cumulative inhibition was seen in many of the microorganisms (6). This work has been extended considerably with the *Escherichia coli* enzyme (24) which is apparently unique in that it exists in two interconvertible forms, the interconversion of which depends on an additional enzyme and a regulatory protein, the latter which itself exists in two forms, catalyzed by two other enzymes (18). The *Bacillus subtilis* (4) and *Neurospora crassa* enzymes (8) have also been the subject of regulatory studies. The rat liver and ovine brain glutamine synthetases are also subject to feedback inhibition by the same compounds effective with the bacterial sources (21, 22). Here, as with the bacterial glutamine synthetase, most inhibitors were more effective if Mn^{2+} replaced Mg^{2+} in the assay medium.

Little information on the regulatory characteristics of higher plant glutamine synthetase was available until recently. Varner and Webster (23) found that the pea seed glutamine synthetase was inhibited by ADP and Pi. Caldos (2) reported that the purified carrot enzyme was slightly inhibited by Pi and a variety of purine and pyrimidine nucleotides, as well as by alanine, glycine, serine, and CAP.

More recently, Kanamori and Matsumoto (7) found that the partially purified rice root glutamine synthetase was inhibited by several purine nucleotides, but amino acids had no effect.

We recently reported the purification of glutamine synthetase (to apparent homogeneity) from pea leaves, and a number of its properties were described (13, 15). The present paper presents the results of studies dealing with the *in vitro* regulation of this enzyme by feedback inhibition.

MATERIALS AND METHODS

The enzyme was purified to at least 90% homogeneity (as judged by disc gel electrophoresis) from 10 to 20-day-old Blue Bantam pea shoots, as previously described (13). The enzyme was stored for periods of 1 to 60 days before use, in the presence of $MgSO_4$ (10 to 15 mM) and either 1 M sucrose or ethylene glycol (20 to 30%, v/v) (13). It was diluted considerably before assay, so that the final Mg^{2+} content during assay (derived from the enzyme) was below 0.1 mM in the cases where the Mn^{2+} biosynthetic assay was used. In some experiments the Mg^{2+} was removed by gel filtration on G-25 Sephadex prior to assay, or by dialysis against 25% ethylene glycol. The enzyme was assayed by procedures described previously (13, 15).

The amino acids and analogues, purine and pyrimidine nucleotides, and carbamyl phosphate were obtained from Sigma Chemical Co. The purine and pyrimidine nucleotides were generally the best grade available.

RESULTS

Inhibition by Purine and Pyrimidine Nucleotides. Since both purine nucleotides and pyrimidine nucleotides require L-glutamine for their synthesis, both classes of compounds can be regarded as end products. These were tested with both Mn^{2+} and Mg^{2+} as the cation sources in the reaction. Only three of a variety of purine and pyrimidine nucleotides had any significant effect on the enzyme activity.

[1] This work was supported by a grant from the National Research Council of Canada.

[2] Present address: Department of Biology, Carleton University, Colonel By Drive, Ottawa, Ontario, Canada.

[3] Abbreviation: CAP: carbamyl phosphate.

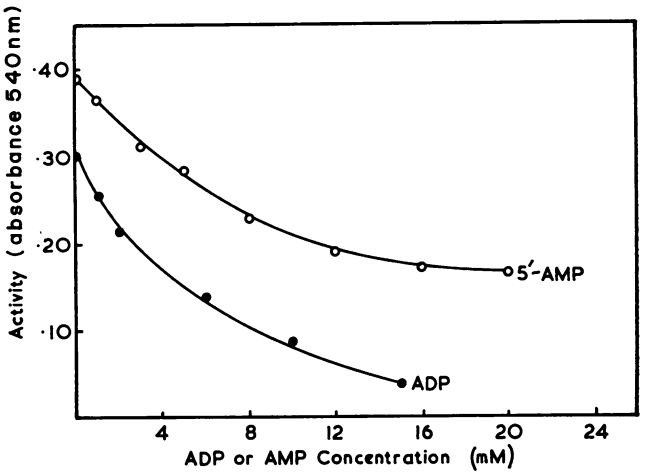

FIG. 1. Effect of ADP and 5'-AMP on Mg^{2+}-dependent glutamine synthetase activity. The reaction contained 6 mM ATP, 20 mM $MgSO_4$, 8 mM NH_2OH, 50 mM L-glutamate, 1 mM DTPA, and 0.1 M Tricine-KOH buffer, pH 7.8. Glutamyl hydroxamate synthesis was assayed.

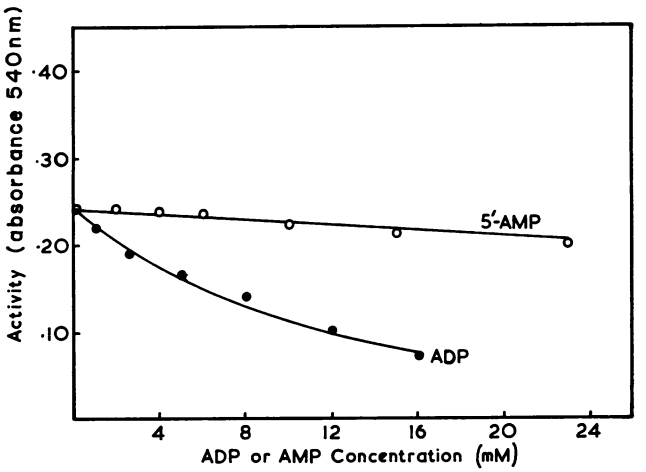

FIG. 2. Effect of ADP and 5'-AMP on Mn^{2+}-dependent glutamine synthetase activity. The reaction contained 6 mM ATP, 8 mM NH_2OH, 30 mM L-glutamate, 3 mM $MnSO_4$ and 0.1 M MES-KOH buffer, pH 6.2. Glutamyl hydroxamate synthesis was assayed. The results are from two separate experiments.

Compounds having no effect (when present equimolar with ATP, or even in 2-fold excess, or with limiting or nonlimiting L-glutamate) included GMP, UMP, CMP, IMP, UDP, GTP, ITP, CTP, and UTP. Adenosine was slightly inhibitory. The three nucleotides which did inhibit were ADP and 5'- and 2'-AMP. Some results with these inhibitors are shown in Figure 1 (2'-AMP caused only two-thirds as much inhibition as 5'-AMP and was not studied further). It was determined that ADP was a competitive inhibitor with respect to ATP and noncompetitive with respect to L-glutamate. Likewise, 5'-AMP was competitive with respect to ATP. The *Ki* values of ADP and 5'-AMP were approximately 1.2 mM and 1.8 mM, respectively.

Where Mn^{2+} was the divalent cation source it was found that 5'-UMP, 5'-IMP, and 5'-CMP caused 7 to 17% stimulation (at 4.6 mM, equimolar with ATP), while adenosine, ITP, UTP, and GTP caused 12%, 14%, 20%, and 5% inhibition, respectively. It is not surprising that ITP, UTP, and GTP cause such limited inhibition, in that they are capable of partially or nearly completely substituting for ATP where Mn^{2+} is the divalent cation (15). ADP and 5'-AMP were not as inhibitory with Mn^{2+} as they were with Mg^{2+} (Fig. 2).

Since ADP and 5'-AMP were inhibitory, it was expected that pea leaf glutamine synthetase would be significantly affected by the "energy charge" (1) as shown in Figure 3. The effect of a decrease in energy charge is more pronounced with Mg^{2+} than with Mn^{2+}. Only a small part of the decrease in activity (with either Mg^{2+} or Mn^{2+}) as the energy charge falls is caused by a reduction of ATP concentration, for the ATP concentrations at energy charges of 0.825 and 0.6 are, respectively, 5.63 mM and 3.29 mM, or 12.5 and 7.3 times the *Km* for ATP under these conditions (15).

Inhibition by Amino Acids. As mentioned earlier, several amino acids can be looked upon as end products whose synthesis requires glutamine. A variety of amino acids were tested to see if they would inhibit the enzyme. The results (in the presence of Mg^{2+}) are summarized in Table I. The amino acids caused only slight inhibition in most cases, even where L-glutamate was limiting. Where Co^{2+} was the divalent cation, the degree of inhibition was either greater or smaller, depending on the amino acid (Table II). It was less with L-alanine and its analogs and serine, but much more with L- and D-histidine. In the case of D-histidine, a brownish-orange color appeared in the assay tubes, indicating the probability of a cobalt-histidine complex, although this did not occur with an equal concentra-

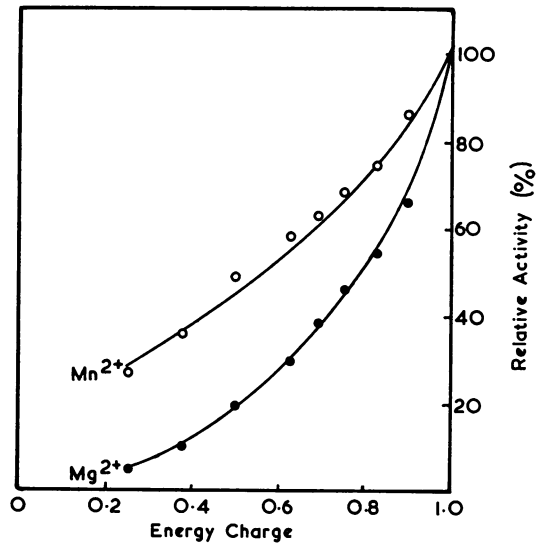

FIG. 3. Effect of energy charge on Mg^{2+}-dependent and Mn^{2+}-dependent glutamine synthetase activity. The desired energy charge was obtained using myokinase by the method of Klugsoyr *et al.* (9). In the Mg^{2+}-dependent assay, 20 μg (16 units) of myokinase was incubated 30 min at 35 C in the presence of the normal glutamine synthetase reaction components (100 mM L-glutamate, Tricine-KOH, pH 7.8, 3 mM NH_4Cl, 1 mM DTPA, and 20 mM $MgSO_4$) plus 8 mM total (ATP plus AMP) in a total volume of 1 ml. The reaction was then started with 0.1 ml of purified glutamine synthetase enzyme and was run for 5 min at 35 C. The modified Boyer Pi assay was employed (13). In the case of the Mn^{2+}-dependent assay, myokinase (20 μg) plus 8 mM (ATP plus AMP) was first incubated for 30 min at 35 C in the presence of 10.8 mM $MgSO_4$ and 0.03 M Tricine-KOH buffer, pH 8.1, in a total volume of 0.25 ml. Then 0.65 ml of glutamine synthetase substrates and buffer was added (0.15 M succinate-KOH buffer, pH 5.2, 60 mM L-glutamate, 6 mM NH_4Cl, and 12 mM $MnSO_4$) and the reaction started with 0.10 ml of purified glutamine synthetase. The reaction was terminated 5 min later and Pi assayed by the modified Boyer technique (13).

Table I. *Effect of Amino Acids on the Mg^{2+}-dependent Activity of Glutamine Synthetase*

The production of glutamyl hydroxamate was assayed. In part A the reaction contained 8 mM ATP, 20 mM $MgSO_4$, 1 mM diethylenetriamine pentaacetic acid, 10 mM mercaptoethanol, 12 mM L-Glu, 8 mM NH_2OH, pH 7.8. In part B the only difference was a higher concentration (36 mM) of L-glutamate.

Treatment	Relative Activity
mM	%
A. Control	100
D-Ala (12)	69
β-Ala (12)	88
L-Ala (12)	86
D-His (12)	92
L-His (12)	86
B. Control	100
Gly (12)	93
L-Arg (12)	94
L-Orn (12)	92
L-Tryptophan (12)	98
L-Citrulline (12)	96
L-Lys (12)	100

Table II. *Effect of Amino Acids on Co^{2+}-dependent Activity of Glutamine Synthetase*

In part A the reaction contained 6 mM ATP, 12 mM L-Glu, 10 mM $CoCl_2$, 1 mM diethylenetriamine pentaacetic acid, 8 mM NH_2OH, pH 6.7. In part B the glutamate concentration was 36 mM. The production of glutamyl hydroxamate was assayed.

Treatment	Relative Activity
mM	%
A. Control	100
D-Ala (12)	78
β-Ala (12)	91
L-Ala (12)	90
D-His (12)	45
L-His (12)	12
L-Tryptophan (12)	88
B. Control	100
L-Ala (12)	94
L-Ser (12)	99
L-Arg (12)	104
L-Citrulline (12)	104
L-Orn (12)	93

tion of L-histidine. Furthermore, increasing the cobalt level (L-histidine, 12 mM, ATP, 6 mM) from 12 mM to 14 mM to 16 mM increased inhibition from 69% to 73% to 76%, respectively, so the inhibition by L-histidine was not due to its chelation of cobalt.

Where Mn^{2+} was employed as the cation source, results were different again. As shown in Table III, L- and D-histidine were not as inhibitory as they were with Co^{2+}, but much more so than with Mg^{2+}. L-Alanine and its analogs were more inhibitory with Mn^{2+} than with either Co^{2+} or Mg^{2+}, as was L-ornithine. In an experiment designed to determine whether the L-histidine inhibition was caused by Mn^{2+} chelation, it was clear that increasing the Mn^{2+} level decreased the per cent inhibition by histidine, but the control activity was also decreased (Fig. 4). The decreased activity in either the absence or presence of L-histidine as Mn^{2+} (total) rises from 3 or 5 mM to 7 to 9 mM is caused by a pronounced shift in pH optimum, which varies considerably with the Mn^{2+} concentration (13). The fact that the decrease in activity with increasing Mn^{2+} is less pronounced

Table III. *Effect of Amino Acids on Mn^{2+}-dependent Activity of Glutamine Synthetase*

The production of glutamyl hydroxamate was assayed. The reaction contained: (part A) 6 mM ATP, 4 mM $MnSO_4$, 8 mM NH_2OH, 4 mM mercaptoethanol, 12 mM L-Glu, pH 6.2; (part B) 8 mM ATP, 4 mM $MnSO_4$, 8 mM NH_2OH, 12 mM L-Glu, pH 6.1; (part C) 6 mM ATP, 3.5 mM $MnSO_4$, 5 mM NH_4Cl, 10 mM L-Glu, pH 6.1.

Treatment	Relative Activity
mM	%
A. Control	100
L-Ala (12)	77
D-Ala (12)	46
β-Ala (12)	72
L-Tryptophan (12)	106
L-His (12)	44
D-His (12)	67
L-Lys (20)	100
B. Control	100
Glyc (12)	82
L-Ser (12)	81
L-Arg (12) or L-citrulline (12)	104
L-Orn (12)	64
C. Control	100
L-His (12)	39
L-3 Methylhistidine (12)	13
L-1 Methylhistidine (12)	70
Aminotriazole (12)	62
Histamine (12)	98
Imidazole acetic acid (12)	94

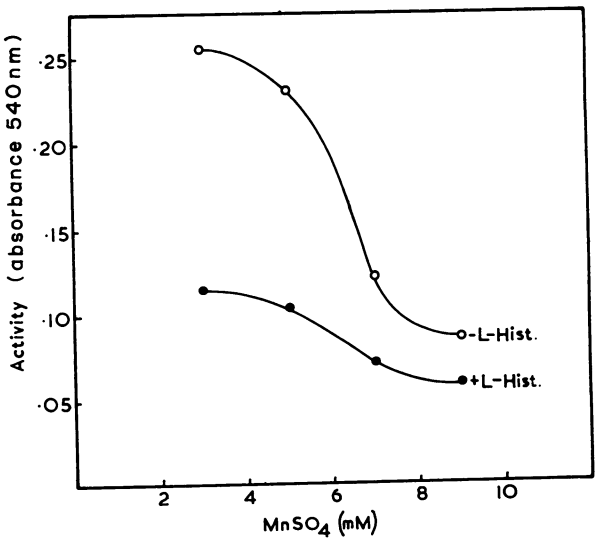

FIG. 4. Effect of $MnSO_4$ concentration on the inhibition of L-histidine. The reaction contained 6 mM ATP, 8 mM NH_2OH, 12 mM L-glutamate, and 0.1 M MES-KOH buffer, pH 6.2. Plus 12 mM L-histidine (●); minus L-histidine (○).

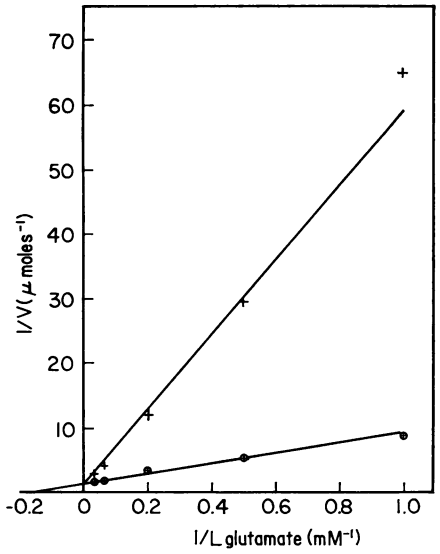

FIG. 5. Inhibition of Mn^{2+}-activatied glutamine synthetase by L-histidine. The reaction contained 6 mM ATP, 8 mM NH_2OH, 4 mM $MnSO_4$, and 0.1 M MES-KOH buffer, pH 6.2. Plus 10 mM L-histidine (+); minus histidine (⊕). Glutamyl hydroxamate synthesis was assayed.

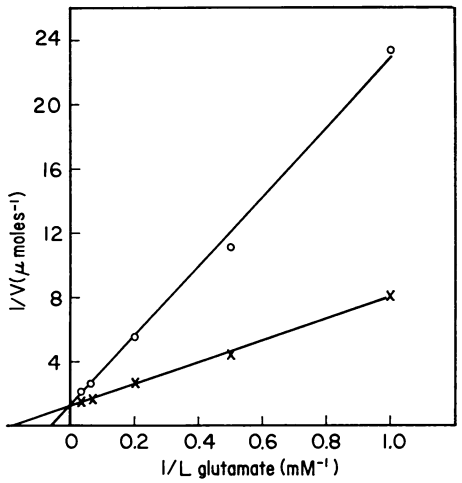

FIG. 6. Inhibition of Mn^{2+}-activated glutamine synthetase by L-ornithine. The reaction contained the same components as in Fig. 5, except ornithine replaced histidine. Minus ornithine (×); plus 20 mM L-ornithine (○).

in the presence of histidine may be due to the complexing of some of the excess Mn^{2+} by L-histidine.

L-Histidine and L-ornithine were competitive inhibitors with respect to L-glutamate, the substrate (Figs. 5 and 6) while L-alanine, glycine, and L-serine were uncompetitive inhibitors with respect to L-glutamate or NH_4Cl (Fig. 7). The inhibition caused by L-histidine was greater at pH 6.1 or 6.2 than it was at pH 5.2, but this may reflect the different ATP and Mn^{2+} concentrations used at the two pH values. The *Ki* value for L-histidine at pH 6.2 (ATP, 6 mM, Mn^{2+}, 3 mM) and pH 5.2 (ATP, 8 mM, Mn^{2+}, 11 mM) were 1.7 to 2.1 mM and 5.9 to 6.5 mM, respectively, while the *Km* values for L-glutamate in the same assays were 6 to 8.7 mM and 7.1 to 8.7 mM, respectively. The *Ki* for L-ornithine at pH 6.2 was 7.8 mM.

L-Histidine inhibition was approximately equal using either NH_2OH or NH_4Cl and using the Pi assay or the glutamyl hydroxamate assay.

A number of analogs of L-histidine were tested to compare their degree of inhibition (Table III). L-3 Methyl histidine was an even more effective inhibitor than L-histidine, while the analogs histamine and imidazole acetic acid caused little or no inhibition. In order to determine if inhibition by the amino acids was additive or cumulative, various combinations were tested, as shown in Table IV. The observed values indicate that inhibition is not additive but more nearly cumulative.

Although L-histidine and L-ornithine were much more inhibitory with Mn^{2+}, they did cause slight inhibition in the Mg^{2+}-dependent assay. The addition of 0.25 mM and 1.25 mM $MnSO_4$ to the Mg^{2+} assay (pH 7.8) did not significantly alter the inhibition caused by L-histidine, L-ornithine, and CAP. However, at pH 6.2 the addition of 0.5 to 1.5 mM $MnSO_4$ (Mg^{2+}, 20 mM) did result in significant inhibition by these compounds (Table V). At this pH, unlike at pH 7.8, L-histidine caused strong stimulation of the Mg^{2+}-dependent activity, if

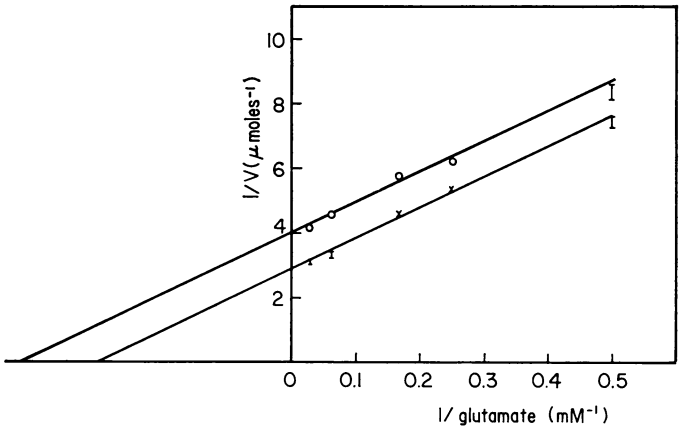

Fig. 7. Inhibition of Mn^{2+}-activated glutamine synthetase by L-serine. The reaction contained 6 mM ATP, 5 mM NH_4Cl, 3 mM $MnSO_4$, and 0.1 M MES-KOH buffer, pH 6.2. Minus serine (×); plus 20 mM L-serine (○). The Pi assay was used (13).

Table IV. *Effect of Combinations of Inhibitors on Mn^{2+}-Dependent Glutamine Synthetase Activity*

The liberation of Pi was assayed. In all cases the reaction contained 3 mM NH_4Cl, 6 mM ATP, 3 mM $MnSO_4$, 0.1 M MES-KOH buffer, pH 6.2, 12 mM L-glutamate, and 0.7 mM mercaptoethanol.

Treatment	Inhibition		
	Observed	Calculated for cumulative	Calculated for additive
	mM	%	
A. L-His (20)	63.5		
L-Orn (30)	53.1		
L-His (20) + L-Orn (30)	73.5	83	116.6
B. L-His (4)	19.4		
L-Orn (8)	18.7		
L-His (4) + L-Orn (8)	31.6	34.5	38.1
C. L-Ala (28)	14.8		
Gly (28)	16.7		
L-Ser (28)	16.0		
Ala (28) + Gly (28)	24	28.8	31.5
Ala (28) + Ser (28)	24	28.4	30.8
Gly (28) + Ser (28)	26.6	30	32.7
Ala + Gly + Ser	34.6	37.4	47.5

Table V. *Effect of Addition of Mn^{2+} in Presence of Mg^{2+} on Inhibition Caused by L-Hist, L-Orn, and Carbamyl Phosphate*

In part A, Pi liberation was assayed, and the reaction contained 8 mM ATP, 0.1 M MES-KOH buffer, pH 6.2, 20 mM $MgSO_4$, 10 mM L-glutamate, 4 mM NH_4Cl. In part B, the glutamyl hydroxamate assay was employed, and the reaction differed in that L-glutamate concn. was 30 mM and NH_2OH was 8 mM. The relative activity with histidine, ornithine, and carbamyl phosphate is the activity relative to controls with the same level of $MnSO_4$.

Treatment	$MnSO_4$	Relative Activity
	mM	%
A. Control	0	100
Control	0.5	210
Control	1.5	173
L-His (10)	0	172
L-His (10)	0.5	79
L-His (10)	1.5	62
L-Orn (20)	0	115
L-Orn (20)	0.5	81
L-Orn (20)	1.5	76
B. Control	0	100
Control	0.5	82
Control	1.5	47
Carbamyl phosphate (10)	0	100
Carbamyl phosphate (10)	0.5	54
Carbamyl phosphate (10)	1.5	47

Table VI. *Effect of Several Inhibitors on Mn^{2+}-dependent Glutamyl-Transferase Activity*

The reaction contained 0.5 mM ADP, 20 mM $NaAsO_4$, 8 mM $MnSO_4$, 25 mM L-glutamate, 10 mM NH_2OH, and 0.08 M MES-KOH buffer, pH 6.6.

Treatment	Relative Activity
mM	%
Control	100
L-Glu (25)	92
L-His (12)	106
L-Orn (12)	91
Carbamyl phosphate (12)	54

L-glutamate was limiting and Mn^{2+} was absent. When Mn^{2+} was present at this pH, L-histidine and L-ornithine did inhibit. Also, Mn^{2+} stimulated the enzyme activity at pH 6.2 (unlike at pH 7.8), but only if L-glutamate was limiting (Table V).

Since L-histidine and L-ornithine appear to compete with L-glutamate, it was of interest to test their effect on the activity of the transfer reaction, where glutamine, not glutamate, was the substrate. These results are shown in Table VI, where neither L-glutamate, L-histidine, or L-ornithine had much effect. This does not prove, however, that L-histidine and L-ornithine do bind at the L-glutamate site.

Other Inhibitors. Inorganic phosphate inhibited the enzyme, and was a noncompetitive inhibitor with respect to ATP and L-glutamate (Fig. 8). The *Ki* value for Pi was 40 mM. Pyrophosphate was a more effective inhibitor than Pi, as shown in Figure 9. These results are difficult to interpret, however, for some precipitation of magnesium pyrophosphate occurred, lowering both the true PPi concentration and the Mg^{2+} concentration. Chelation of Mg^{2+} is not the main reason for the PPi inhibition in this experiment, for at 6 mM PPi, even if all of the PPi were complexed to Mg^{2+} there would still be a 6.2 mM excess of free Mg^{2+} present (not complexed to ATP or PPi), which is enough to ensure at least 90% V_{max} (15).

Glucosamine 6-P, which is derived from glutamine, caused only 12% inhibition at 12 mM in (ATP, 6 mM, L-glutamate, 50 mM, NH_2OH, 8 mM, $MgSO_4$, 20 mM). With Mn^{2+}, glucosamine 6-P had no effect. Asparagine and glutamine caused only slight inhibition at concentrations up to 81 mM, while 33 mM aspartate caused 27% inhibition (12 mM L-glutamate was limiting, in these cases).

Carbamyl phosphate caused significant inhibition with Mn^{2+} but much less inhibition with Mg^{2+}, where 5 mM CAP and 10 mM CAP caused only 8% and 22% inhibition, respectively (ATP, 6 mM, $MgSO_4$, 20 mM, L-glutamate, 12 mM, and NH_2OH, 10 mM). The inhibition by CAP was not due to use of the lithium salt, since this cation caused only 8% inhibition

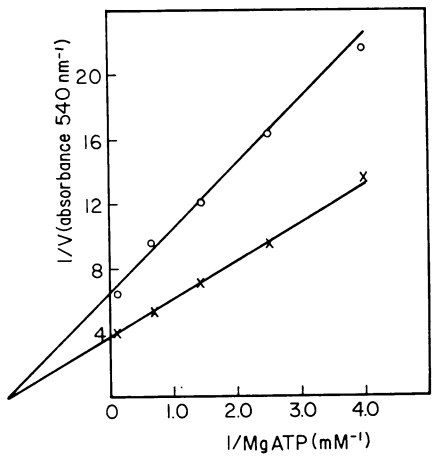

FIG. 8. Inhibition of Mg^{2+}-activated glutamine synthetase by Pi. The reaction contained 4 mM NH_2OH, 60 mM L-glutamate, 45 mM $MgSO_4$, and 0.1 M Tricine-KOH buffer. Minus Pi (×); plus 30 mM Pi (○). Glutamyl hydroxamate synthesis was assayed.

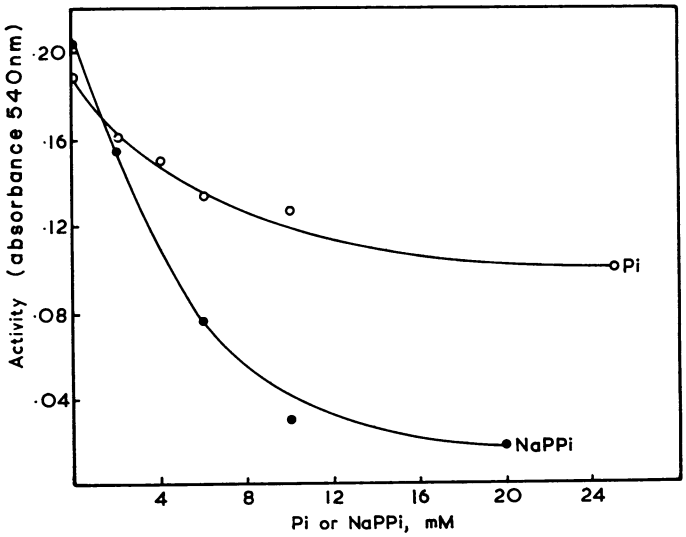

FIG. 9. Effect of Pi and PPi on glutamine synthetase activity. Pi curve: the reaction contained 8 mM ATP, 50 mM L-glutamate, 8 mM NH_2OH, 1 mM diethylenetriamine pentaacetic acid, 40 mM $MgSO_4$, and 0.1 M Tricine-KOH buffer, pH 7.8. PPi curve: as above, but $MgSO_4$ = 20.2 mM. In both cases, glutamyl hydroxamate was assayed.

at 20 mM. Carbamyl phosphate did not appear to be competitive with NH_2OH, ATP, or L-glutamate. In fact, as L-glutamate concentration was increased from 4 mM to 45 mM the inhibition caused by 14.4 mM CAP increased from 40% to 55%, when assayed at pH 5.2 (succinate-NaOH buffer, 0.15 M, pH 5.2, $MnSO_4$, 10 mM, ATP, 8 mM, NH_2OH, 10 mM). Under similar assay conditions increasing $MnSO_4$ from 7 mM to 13 mM or 16 mM increased inhibition by 14.4 mM CAP from 31% to 51%. If assayed at pH 5.2, where Mn^{2+} was always 3 mM in excess of ATP level, then as ATP concentration increased from 0.5 mM to 8 mM the inhibition by CAP rose from 41% to 52%, indicating that ATP must in some manner increase the sensitivity to CAP.

Although it is possible that CAP is not inhibiting directly, but through a product of degradation, the major degradation product, sodium cyanate (24), caused no inhibition at a concentration of 5 mM. Furthermore, the CAP was made up immediately prior to assay, added just before the enzyme, and the assay period was only 5 min, so it is doubtful that much degradation occurred in this time.

DISCUSSION

Of a variety of purine and pyrimidine nucleotides tested, only ADP and 5'-AMP caused significant inhibition of pea leaf glutamine synthetase, and this inhibition was competitive with ATP. While 5'-AMP is inhibitory to glutamine synthetase derived from a variety of other organisms, including *E. coli* (24), *B. Subtilis* (4), *Neurospora crassa* (8), carrot (2), rice (7), and pea seeds (23), apparently the mechanism of this inhibitor varies. With the *E. coli* enzyme, 5'-AMP is a noncompetitive inhibitor with respect to ATP or glutamate, but no data are given with respect to the other sources above. ADP also inhibits the glutamine synthetase from the above sources, and is a more effective inhibitor than AMP. In those cases where it has been studied, it appears to be a competitive inhibitor with respect to ATP (24). It is interesting to note that in the present study 5'-AMP and ADP were much more inhibitory where Mg^{2+} was used than with Mn^{2+}. Since the enzyme is inhibited by ADP and AMP, its activity is modulated by the "energy charge" in a manner similar to many other ATP-utilizing enzymes (1), and the steepness of the slope at higher energy charges is greater with Mg^{2+} than with Mn^{2+} (Fig. 3).

Another product of the reaction, Pi, is also inhibitory, but less so than 5'-AMP. Phosphate inhibition has been reported with the carrot enzyme (2), the pea seed enzyme (23), and the rat liver enzyme (22), although the nature of the inhibition was not reported. The inhibition by PPi in the present study is even greater than that caused by ADP, and it is probably competitive with ATP. Pyrophosphate inhibition of this enzyme from other sources has not been reported to our knowledge.

The lack of a significant degree of inhibition by other purine and pyrimidine nucleotides is unlike the situation existing for glutamine synthetase from many other sources. Glutamine synthetase from most microorganisms is inhibited by CTP or GTP, and these compounds were more inhibitory in the presence of Mn^{2+} than with Mg^{2+} (4, 6, 8). However, these compounds usually had to be present at concentrations of over 2 mM to cause more than 25% inhibition. Glutamine synthetase from carrot is inhibited by CDP, CTP, UDP, UTP, GDP, GTP, using Mg^{2+} (2). Strong noncompetitive inhibition of the rice enzyme by GTP was observed (Mg^{2+} in assay) (7), but since the enzyme was preincubated with GTP (10 min, 35 C) before assay perhaps GTP was simply inactivating the enzyme.

The inhibition observed with alanine, glycine, and serine has been seen with glutamine synthetase from many sources, including *E. coli* (24), rat liver (21, 22), *B. subtilis* (4), and carrot (2). Little or no inhibition by these compounds was seen in the ovine enzyme (22), *N. crassa* (8), or rice (7). This inhibition is usually only weak, but is much greater in the presence of Mn^{2+} than Mg^{2+} (4, 21, 22, 24), as observed in the present study. Tate *et al.* (22) reported that the pea seed enzyme (glutamyl transferase activity) was inhibited 27 to 53% by L-serine, glycine, and L-alanine (Mn^{2+}-dependent activity), where these amino acids were present at 20 mM, which is similar to our results. In most cases, the nature of the binding site for these compounds is not known. In the work described here they do not compete with L-glutamate or NH_4^+. It is interesting to note that β-alanine is a more effective inhibitor than L-alanine in the pea leaf enzyme as well as with the rat liver and ovine brain enzymes (22), but not with *E. coli* (24).

Although arginine synthesis requires glutamine, arginine does not inhibit the pea leaf enzyme, nor those from other sources tested (24). Of other intermediates in the urea cycle, only L-ornithine (Mn^{2+} assay) caused significant inhibition in the present work, and it appeared to compete with L-glutamate. To our knowledge this compound was not tested as an inhibitor with enzymes from other sources.

Carbamyl phosphate requires glutamine for its synthesis, and is required for arginine biosynthesis. This compound did inhibit significantly at sufficiently high concentrations (Mn^{2+}-dependent activity) and was noncompetitive with respect to glutamate, ATP, or NH_2OH. CAP has been found to inhibit glutamine synthetase from most organisms, and usually is much more inhibitory where Mn^{2+} replaces Mg^{2+} (4, 6, 11, 22, 24). It was not tested as an inhibitor with the rice enzyme (7), but 20 mM CAP did cause 50% inhibition with the carrot enzyme (Mg^{2+}-dependent activity) (2). In the case of *E. coli*, CAP was not competitive with any of the substrates (24). In all systems where CAP has been found to be an inhibitor, relatively high amounts (5 to 10 mM) are required for about 50% inhibition. It is significant that in the present study the two compounds (CAP and ornithine required for citrulline and, ultimately, arginine synthesis act as inhibitors.

L-Histidine, also derived in part from glutamine, was among the most effective inhibitors tested in the present work. As with all other inhibitors except ADP and AMP, histidine was much more inhibitory in the presence of Mn^{2+} than with Mg^{2+}. This is again similar to the case for the enzyme from other organisms (4, 6, 8, 22, 24), although the pea leaf enzyme appears to be more sensitive to histidine inhibition than those of other sources. The rat liver enzyme was inhibited by histidine only in the presence of Mn^{2+}, and histidine was competitive with L-glutamate (22). The rice enzyme (Mg^{2+}-dependent activity) was not inhibited by 20 mM histidine, (7), while the carrot enzyme (Mg^{2+}-dependent activity) was actually stimulated 15% by 30 mM histidine (2). The fact that inhibition by histidine did not occur where the glutamyl transfer assay was used suggests that it does not compete with glutamine, as opposed to glutamate. This was analogous to the situation observed with ornithine, another glutamate competitor.

It should be emphasized that the "competitive inhibition" caused by histidine and ornithine does not in itself prove they are truly competing with L-glutamate for the glutamate binding site—alternative explanations are possible.

At this point it is difficult to interpret the *in vivo* significance, if any, of the inhibition caused by glycine, alanine, serine, CAP, ornithine, and histidine. First, only with Mn^{2+} do they inhibit significantly and whether Mn^{2+} (or MnATP) ever replaces Mg^{2+} (or MgATP) in this reaction in plant cells is unknown and is perhaps unlikely owing to the much greater *in vivo* concentrations of Mg^{2+} than Mn^{2+} (12). Second, the concentrations

of the above inhibitors needed for 50% inhibition are high: 1.7 to 6.5 mM for histidine, 10 to 12 mM for CAP, 7.8 mM for ornithine, and over 40 mM for glycine, serine, and alanine. It can be questioned whether these intermediates ever accumulate in such amounts. The cumulative nature of these inhibitors could result in significant inhibition where most or all of them were present simultaneously in more moderate amounts. Actually, with the glutamine synthetase of most organisms all of the individual inhibitors mentioned previously must be present in millimolar concentrations for significant inhibition to occur, and it is not yet clear whether such regulation occurs *in vivo*. With respect to the present work, it should be pointed out that even though ornithine and histidine must be present at levels of about 2 to 8 mM for 50% inhibition, their K*i* values are about equal to (for ornithine) or $\leqslant$ half (for histidine) the K*m* for glutamate, so that if the histidine and ornithine levels *in vivo* are only half the glutamate level, significant inhibition may occur.

It is more likely that pea leaf glutamine synthetase activity is regulated by the energy charge of the cell. With Mg^{2+}, the relative activity at an energy charge of 0.8 and 0.5 were 53% and 20%, respectively, while with Mn^{2+} the relative activities were 73% and 49%, respectively. It appears that a significant amount of glutamine synthetase is present in the chloroplasts (14) where the energy charge in the light ranges from 0.66 to 0.76 in isolated illuminated spinach chloroplasts but only 0.50 to 0.58 in isolated nonilluminated chloroplasts (10, 17). Recently, Canvin and Atkins (3) showed that barley and bean leaves incorporated ammonia ($^{15}NH_4^+$) into amino acids about five times faster in the light than in the dark, an observation which supports the effect of energy change on glutamine synthetase.

Acknowledgment—We are indebted to Miss Nancy Smith for her able technical assistance.

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