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Regulatory properties of rice leaf glutamine synthetase by amino acids and nucleotides[1,2]

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Abstract. Various L-amino acids and nucleotides were used to inhibit the Mg^{2+}-supported biosynthetic activity of rice leaf glutamine synthetases GSI and GSII. The most potent inhibitors are glycine, alanine, aspartic acid, serine, AMP and ADP. When increasing concentration of the six inhibitors to saturating level, the remaining percent activity of GSI and GSII decreased to a finite value indicating partial inhibition. The results was further confirmed by fractional inhibition analysis and inhibition kinetics. The combined effects obtained with all 15 possible pairs of these six inhibitors on the activities of GSI and GSII showed that 6 pairs are cumulative inhibition and 9 pairs are antagonistic inhibition on GSI, whereas 14 pairs are cumulative inhibition and one pair is antagonistic inhibition on GSII. The results indicated that both GSI and GSII possess separate binding sites for each of these six inhibitors, but the interactions of these inhibitor sites on the surface of both enzymes are not identical. When glutamate was the varied substrate, glycine and alanine were partial uncompetitive inhibitors, while serine and aspartic acid were partial noncompetitive inhibitors of GSI; however, glycine, alanine and aspartic acid were partial mixed-type inhibitors, and serine was partial noncompetitive inhibitor of GSII. When ATP was the varied substrate, AMP and ADP were partial competitive inhibitors of both GSI and GSII.

Key words: Amino acid; Glutamine synthetase; Inhibition; Nucleotide; *Oryza sativa;* Regulatory properties.

Introduction

The rate of metabolic processes depends on the amount and catalytic efficiency of the enzymes concerned. The end-product control of the synthesis and activity of biosynthetic enzymes is recognized as an important way in regulation of cellular growth processes. Glutamine synthetase [L-glutamate: ammonia ligase (ADP-forming); EC 6.3.1.2] occupies a key position in a highly branched metabolic pathway to catalyze the first reaction in the complex branched pathway leading to the synthesis of a number of basic products such as amino acids, carbamyl phosphate, purine and pyrimidine nucleotides (Greenberg, 1969; Hubbard and Stadtman, 1967), and its activity is under rigorous cellular control. Hubbard and Stadtman (1967) mentioned that the glutamine

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synthetase from different sources is really inhibited by a variety of nucleotides, amino acids and some cellular nitrogenous constituents including AMP, CTP, histidine, tryptophan, glycine, alanine, glucosamine 6-phosphate, and carbamyl phosphate. Because glutamine serves diverse functions in different cells, a study of the regulatory mechanism of the glutamine synthetase isolated from different sources is of great significance. Although glutamine synthetase from plant sources has been studied in some detail, little information is available for the regulatory properties of this enzyme. Some investigators reported that glutamine synthetase from rice roots (Kanamori and Matsumoto, 1972), green pea seeds (Kingdon, 1974), pea leaves (O'Neal and Joy, 1975), mung bean seedlings (Steethalakshmi and Appji Rao, 1979), soybean root nodules (McParland *et al.*, 1976), and blue green algae (Sawhney and Nicholas, 1978; Stacey *et al.*, 1979; Tuli and Thomas, 1980) was subjected to feedback inhibition by some amino acids and nucleotides. In rice leaves, two forms of glutamine synthetases GSI and GSII were isolated and their properties were also studied in some detail (Hirel and Gadal, 1980; Iyer *et al.*, 1981; Yuan and Hou, 1987); however, the kinetic features of the regulation of these enzyme activities have not been thoroughly studied. In this paper, we describe the inhibitory effect of glycine, alanine, aspartic acid, serine, AMP and ADP on the activities of rice leaf glutamine synthetases GSI and GSII including the mode of inhibition, interactions between inhibitor sites and kinetic features of inhibition. The differences between these two enzymes are compared and discussed.

Materials and Methods

Materials

Rice (*Oryza sativa* L. *japonica* cv. Hsinchu 56) plants were grown in paddy field at the experimental farm of the Institute of Botany, Academia Sinica. Field management including basal and top dressings of fertilizer and irrigations followed the general method used by farmers. Rice leaves were collected at the tillering stage. After washing with distilled water, leaves were sliced to small pieces and stored at −60°C before use. The activity of glutamine synthetase was stable for at least three months at −60°C. The amino acids and nucleotides were purchased from Sigma (St. Louis, Mo., U. S. A.). All other chemicals used in this study were of analytical grade from Merck (Darmstadt, FRG) or Wako Puro Chemical Industries (Tokyo, Japan).

Enzyme Preparation

Frozen rice leaves were ground to powder with liquid nitrogen using a mortar and a pestle, and all further operations were carried out at 4°C. Rice leaf glutamine synthetases GSI and GSII were purified to homogeneity by the method described previously (Yuan and Hou, 1987). The procedure involved ammonium sulfate precipitation, gel filtration through a Sepharose 4B column, ion exchange chromatography on DEAE-Sephacel column to separate GSI and GSII, affinity chromatography on 2' 5' ADP-Sepharose 4B column and hydroxyapatite adsorption. GSI and GSII were finally purified to about 500-fold and 60-fold, respectively. These enzymes were stable for several weeks when stored at 4°C.

Enzyme Assay

The biosynthetic activity of glutamine synthetase was assayed based on the release of inorganic phosphate in the presence of ammonium chloride according to the procedure of Shapiro and Stadtman (1970). The assay mixture in a final volume of 0.4 ml 50 mM imidazole-HCl buffer (pH 7.0) containing 7.5 mM

ATP, 100 mM sodium glutamate, 50 mM NH_4Cl, 50 mM $MgCl_2$ and appropriate amount of enzyme solution. The reaction was started by adding the enzyme solution, and the enzyme solution was omitted in the blank test. After incubation at 30°C for 15 minutes, the reaction was stopped by adding 3.6 ml of ferrous sulfate reagent (0.8% $FeSO_4 \cdot 7H_2O$ in 0.015 N H_2SO_4, prepared freshly), followed by adding 0.3 ml of ammonium molybdate reagent [6.6% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 7.5 N H_2SO_4]. After several minutes, the absorbance at 660 nm was measured. The sodium dihydrogenphosphate dihydrate was used as standard. One unit of glutamine synthetase is defined as the amount of enzyme catalyzing the release of 1.0 μmole inorganic phosphate per minute at 30 °C.

Inhibition Studies

Inhibitor levels were varied by the folllowing technique: a single assay reaction mixture was prepared and divided into two parts, with the inhibitor added to one of these two parts at its maximal concentration. These two parts of reaction mixture were then mixed in different proportions to a fixed final volume (0.3 ml) to make a series of inhibitor levels in the reaction mixture.

In all cases, the fractional inhibition, i, was defined as $(Vo-Vi)/Vo$, where Vo and Vi are defined as the reaction velocities at zero and at finite level of the inhibitor being varied, respectively. Equations relating i to inhibitor concentrtation, [I], have been derived and discussed by Webb (1963), and it has been shown that one can plot $1/i$ versus $1/[I]$, in analogy to a Lineweaver-Burk plot. In such double reciprocal plots, if $1/i_{max} = 1.0$, this implies 100% inhibition at saturating level of inhibitor, whereas partial inhibitions are indicated by $1/i_{max} > 1.0$. In the experiments involving pairs of inhibitors bound to a substrate, each inhibitor was used at a concentration that gave i values in the range between 0.1 to 0.3. Under these conditions, one obtains significant differences between the calculated values for cumulative and additive models of inhibition.

Protein Determination

The protein content was estimated by the dye-binding method of Bradford (1976). Crystalline bovine serium albumin was used as the standard.

Results

Inhibition by Amino Acids

Amino acids can be viewed as end products

Table 1. *Effect of amino acids on glutamine synthetases GSI and GSII from rice leaves*

The biosynthetic activity was determined from the rate of Pi production. The concentration in mM of each of the indicated amino acids in the reaction mixture is shown in parentheses. In control assays, 936 nmole Pi and 985 nmole Pi were produced by GSI and GSII in 15 minutes, respectively.

Amino acid	% Activity remaining		% Inhibition	
	GS I	GS II	GS I	GS II
None	100	100	0	0
L-Alanine (25)	77	85	23	15
L-Arginine (25)	97	99	3	1
L-Aspartic acid (25)	62	76	38	24
L-Glutamine (25)	94	95	6	5
L-Glycine (25)	67	89	33	11
L-Histidine (25)	93	97	7	3
L-Isoleucine (17)	95	97	5	3
L-Leucine (12)	98	98	2	2
L-Lysine (25)	96	101	4	—
L-Methionine (25)	98	103	2	—
L-Phenylalanine (12)	101	103	—	—
L-Proline (25)	94	93	6	7
L-Serine (25)	79	85	21	15
L-Threonine (25)	96	98	4	2
L-Tryptophan (6)	98	100	2	—
L-Valine (25)	98	100	2	—
L-Citruline (25)	91	96	9	4
L-Ornithine (25)	94	99	6	1

of glutamine metabolism. Various L-amino acids were tested to see if they would inhibit Mg^{2+}-supported biosynthetic activity of rice leaf glutamine synthetases GSI and GSII. The results are summerized in Table 1. Among the amino acids tested, alanine, aspartic acid, glycine and serine significantly inhibited the activity of both GSI and GSII. Glutamine, histidine, isoleucine, proline, citruline and ornithine were slightly inhibitory to GSI; and glutamine and proline were also slightly inhibitory to GSII. Other amino acids such as arginine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine were almost ineffective to both GSI and GSII.

The effects of different concentrations of alanine, aspartic acid, glycine and serine on the activities of GSI and GSII were examined. The results are shown in Fig. 1. The plots of the percent activity remaining against amino acid concentrations showed hyperbolic and the residual activity reached a finite value indicating partial inhibition. To understand whether these amino acids at their saturating inhibitory concentrations resulted in a complete inhibition of enzyme activity, the data was further plotted by the double reciprocal plots of the fractional inhibition, *i*, versus concentra-

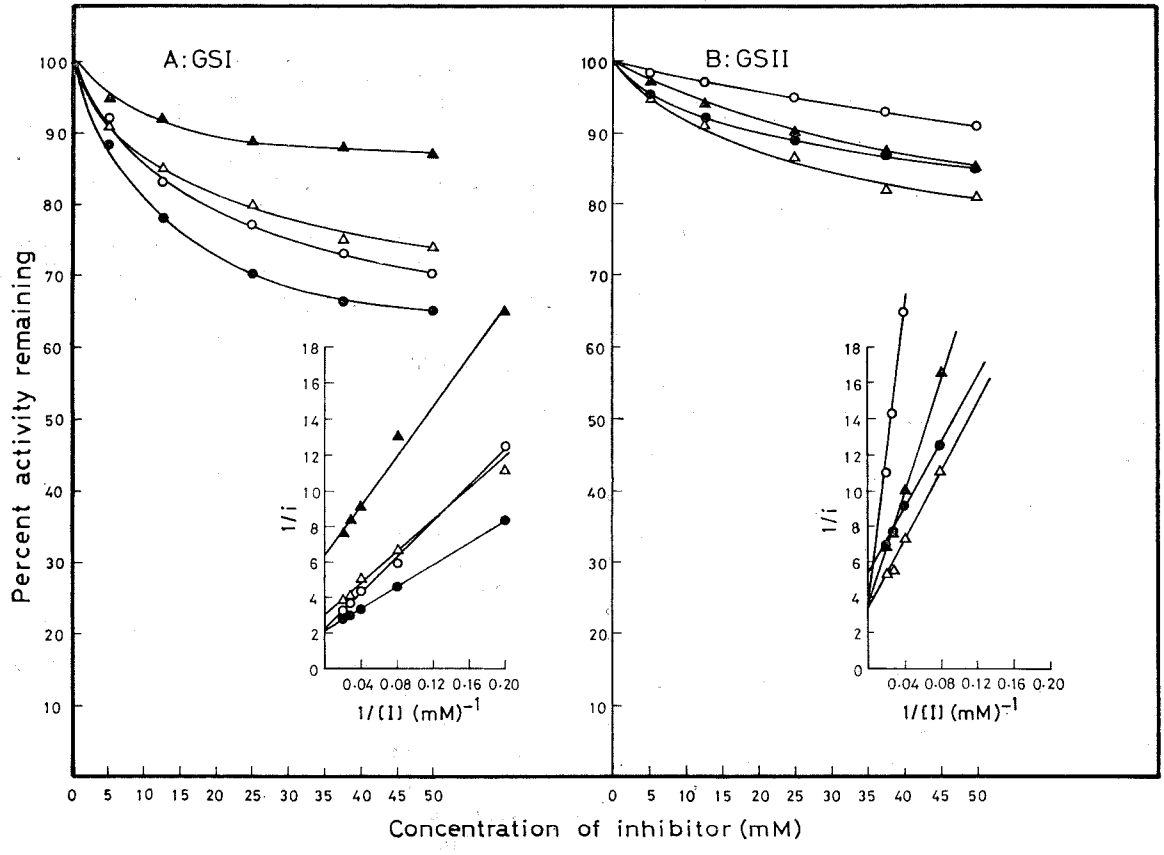

Fig. 1. Effect of glycine (●), alanine (○), serine (▲) and aspartic acid (△) on the activity of glutamine synthetases GS I (A) and GS II (B) from rice leaves. Insets: Plot of $1/i$ versus $1/[I]$, where i represents the fractional inhibition calculated according to the equation $i=(Vo-Vi)/Vo$, Vo is the enzyme activity in the absence of the inhibitor, and Vi represents the activity in the presence of the inhibitor. [I] denotes the concentration of inhibitors. The biosynthetic activity was determined from the rate of Pi production.

tions of amino acids, [I], (insets in Fig. 1). Since ordinate intercept values for these four amino acids were greater than unity, these amino acids only partially inhibited the activities of these enzymes (Webb, 1963).

Inhibition by Nucleotides

Purine and pyrimidine nucleotides can also be regarded as end products of glutamine metabolism. The nucleotide specificity of Mg^{2+}-supported biosynthetic activity of rice leaf glutamine synthetases GSI and GSII is shown in Table 2. The data presented are from experiments in which optimal concentration of 50 mM $MgCl_2$ was used for the Mg^{2+}-supported biosynthetic assay. The effects are therefore probably not due to lowering the concentration of Mg^{2+} by chelation with the added nucleotides.

Table 2. *Effect of nucleotides on the activity of glutamine synthetases GSI and GSII from rice leaves*

The biosynthetic activity was determined from the rate of Pi production. The reaction mixture in a final volume of 0.4 ml 50 mM imidazole-HCl buffer (pH 7.0) contained 7.5 mM ATP, 100 mM sodium glutamate, 50 mM NH_4Cl, 50 mM $MgCl_2$, 10 mM of each of the indicated nucleotides and appropriate amount of enzyme solution. In control assays 762 nmole Pi and 993 nmole Pi were produced by GSI and GSII in 15 minutes, respectively.

Nucleotide (10 mM each)	% Activity remaining		% Inhibition	
	GS I	GS II	GS I	GS II
None	100	100	0	0
AMP	73	74	27	26
ADP	69	65	31	35
UMP	94	94	6	6
UDP	104	93	—	7
UTP	112	105	—	—
CMP	110	108	—	—
CDP	109	101	—	—
CTP	108	110	—	—
GMP	89	88	11	12
GDP	125	116	—	—
GTP	120	112	—	—

Among the nucleotides tested, only AMP and ADP substantially inhibited the biosynthetic activities of GSI and GSII. UMP and GMP were silghtly inhibitory to both GSI and GSII. UDP and CMP were almost ineffective to GSI, however, UDP was slightly inhibitory to GSII and CMP was slightly stimulative to GSII. UTP and CDP were almost ineffective to GSII, whereas, both nucleotides were slightly stimulative to GSI. CTP, GDP and GTP were slightly stimulative to both GSI and GSII.

The effects of different concentrations of AMP and ADP on the activities of GSI and GSII were also examined. The results are shown in Fig. 2. The plots of percent activity remaining against AMP and ADP concentrations also showed hyperbolic and the residual activity reached a finite value. The double reciprocal plots of the fractional inhibition, i, versus concentrations of AMP and ADP, [I] (insets in Fig. 2), also showed greater than unity for ordinate intercept values, indicating that AMP and ADP partially inhibited the activities of these enzymes (Webb, 1963).

Site Interactions between Inhibitors

In order to determine the extent to which these inhibitors are independent in their action, the effects of virtually all possible combinations of the most potent amino acids and nucleotides including glycine, alanine, aspartic acid, serine, AMP and ADP, in pairs and then more than two inhibitors, were considered. The combined effects obtained with 19 combinations of these six inhibitors on the biosynthetic activity of GSI and GSII are shown in Table 3 and Table 4, respectively. From the level of inhibition observed with each inhibitor alone, calculations were made to predict percent inhibition with two or more inhibitors present, depending upon whether their action was additive, synergestic, cumulative, antagonistic or allosteric site interaction (Woolfolk and

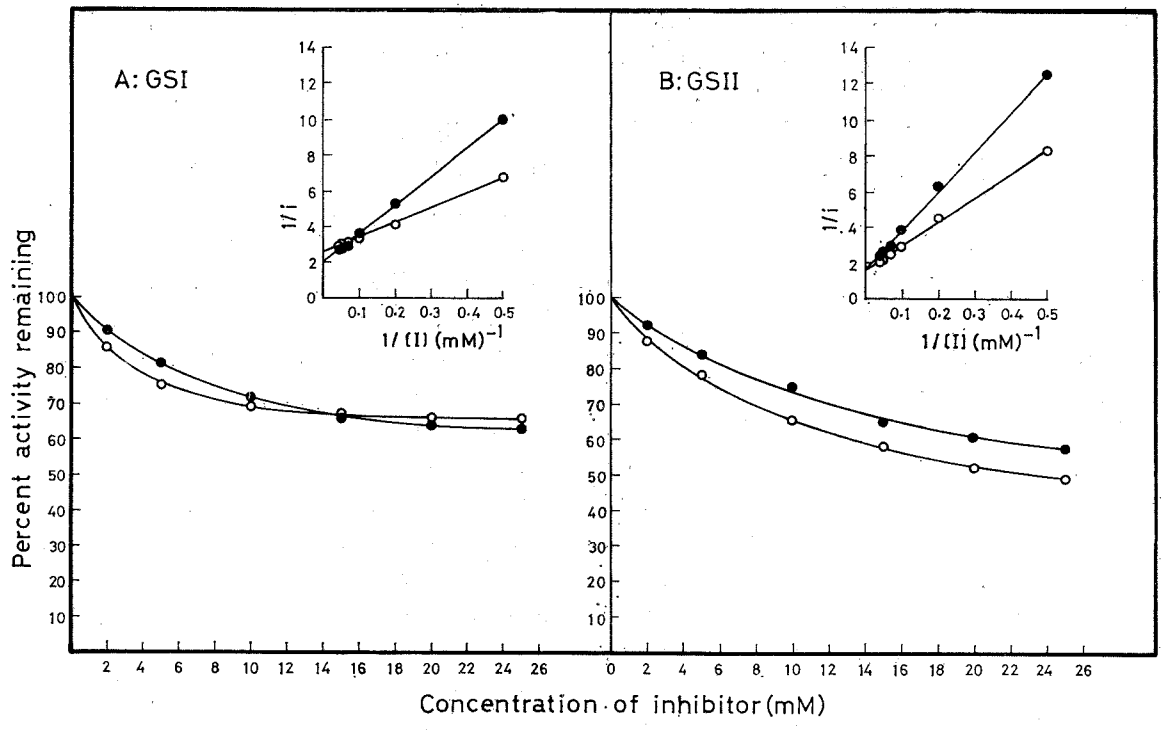

Fig. 2. Effect of AMP (●) and ADP (○) on the activity of glutamine synthetases GS I (A) and GS II (B) from rice leaves. Insets: Plot of $1/i$ versus $1/[I]$, where i represents the fractional inhibition calculated according to the equation $i=(Vo-Vi)/Vo$, Vo is the enzyme activity in the absence of the inhibitor, and Vi represents the activity in the presence of the inhibitor. [I] denotes the concentration of inhibitors. The biosynthetic activity was determined from the rate of Pi production.

Stadtman, 1967). A variety of effects was observed, but additive, synergestic and allosteric site inhibitions were virtually absent in both cases of GSI and GSII. Among 15 pairs (from experiment 1 to 15) of combined inhibitors, the combined inhibitory effect of each pair on GSI activity showed that 6 pairs were cumulative inhibition and 9 pairs were antagonistic inhibition (Table 3); however, the inhibitory effect on the activity of GSII showed that 14 pairs were cumulative inhibition and only one pair was antagonistic inhibition (Table 4). The results indicated that GSI and GSII molecules may possess separate binding sites for each of these six inhibitors, but the distribution of inhibitor sites on the surface of both enzymes are not identical.

Kinetics of Inhibition

Kinetics of inhibition of rice leaf glutamine synthetase GSI activity by glycine, alanine, aspartic acid, serine, AMP and ADP had been studied. Partial uncompetitive inhibition of GSI by glycine and alanine is depicted in Fig. 3A and 3B which represent the double reciprocal plots when glutamate concentration was varied from 1 to 100 mM at three different concentrations of glycine and alanine. According to the expressions for partial uncompetitive inhibition (Segel, 1975):

$$\frac{1}{V_{max_i}}=\frac{1}{V_{max}}\cdot\frac{1+[I]/\alpha Ki}{1+[I]/Ki}$$

and

$$-\frac{1}{Ks_{app}}=-\frac{1}{Ks}\cdot\frac{1+[I]/\alpha Ki}{1+[I]/Ki}$$

where V_{max} is the limiting maximal velocity, V_{max_i} is the limiting velocity in the presence

Table 3. *Combined effects of glycine, L-alanine, L-serine, L-aspartic acid, AMP and ADP on the activity of glutamine synthetase GSI*[a]

Expt	Inhibitor[d]	% Inhibition				Pattern of inhibition[e]
		Observed	Calculated for			
			Cumulative inhibition[b]	Additive inhibition	Single allosteric site	
1	Gly(32)+Ala(21)[e]	37	46	53	32	Ant.
2	Gly(32)+Ser(22)	38	47	54	32	Ant.
3	Gly(32)+Asp(45)	49	63	77	45	Ant.
4	Gly(32)+AMP(25)	39	49	57	32	Ant.
5	Gly(32)+ADP(28)	43	51	60	32	Cuml.
6	Ala(21)+Ser(22)	35	38	43	22	Cuml.
7	Ala(21)+Asp(45)	50	57	66	45	Ant.
8	Ala(21)+AMP(25)	35	41	46	25	Cuml.
9	Ala(21)+ADP(28)	38	43	49	28	Cuml.
10	Ser(22)+Asp(45)	48	57	67	45	Ant.
11	Ser(22)+AMP(25)	36	42	47	25	Cuml.
12	Ser(22)+ADP(28)	41	44	50	28	Cuml.
13	Asp(45)+AMP(25)	49	59	70	45	Ant.
14	Asp(45)+ADP(28)	48	60	73	45	Ant.
15	AMP(25)+ADP(28)	33	46	53	28	Ant.
16	Gly(32)+Ala(21)+Ser(22)	48	58	75		
17	Gly(32)+Ala(21)+Ser(22)+Asp(45)	51	77	120		
18	Gly(32)+Ala(21)+Ser(22)+Asp(45)+AMP(25)	56	83	145		
19	Gly(32)+Ala(21)+Ser(22)+Asp(45)+AMP(25)+ADP(28)	62	88	173		

[a]The biosynthetic activity was determined from the rate of Pi production.
[b]Values for cumulative inhibition were calculated by the procedure of Woolfolk and Stadtman (1967) according to the formula $Xi+(Yi/100)(100-Xi)$, where Xi and Yi are the values of percent inhibition observed with inhibitors X and Y alone, respectively.
[c]Ant.=Antagonistic inhibition, Cuml.=Cumulative inhibition.
[d]Abbreviations of inhibitors: Gly=glycine, Ala=L-alanine, Ser=L-serine, Asp=L-aspartic acid, AMP=adenosine 5′-phosphate, ADP=adenosine 5′-diphosphate. The concentration of inhibitors used was (in mM): Gly(25), Ala(25), Ser(25), Asp(25), AMP(5), ADP(5).
[e]Values in the parentheses are percent inhibition by individual inhibitor.

of a given inhibitor concentration, Ks is the dissociation constant of the enzyme-substrate complex, Ks_{app} is the apparent Ks in the presence of a given inhibitor concentration, [I] is the given inhibitor concentration, Ki is the dissociation constant of the enzyme-inhibitor complex, and α is the interaction constant for the alternation of Ks due to bound inhibitor. The values of $\alpha=0.463$ and $Ki=21.563$ mM were calculated from Fig. 3A when glycine was used as the inhibitor; and the values of $\alpha=0.534$ and $Ki=25.480$ mM were calculated from Fig. 3B when alanine was the inhibitor. Replots of 1/V-axis intercept of the reciprocal plots versus the concentrations of glycine and alanine were hyperbolic as shown in insets of Fig. 3A and 3B, respectively. Partial noncompetitive inhibition of GSI by serine and aspartic acid is depicted in Fig. 4. Double reciprocal plots of velocity versus the concentration of glutamate at three concentrations of serine and aspartic acid are shown in Fig. 4. By using the expres-

Table 4. *Combined effects of glycine, L-alanine, L-serine, L-aspartic acid, AMP and ADP on the activity of glutamine synthetase GSII*[a]

Expt	Inhibitor[d]	% Inhibition				Pattern of inhibition[e]
		Observed	Calculated for			
			Cumulative inhibition[b]	Additive inhibition	Single allo-steric site	
1	Gly(42)+Ala(32)[e]	58	61	74	42	Cuml.
2	Gly(42)+Ser(39)	60	65	81	42	Cuml.
3	Gly(42)+Asp(51)	69	72	93	51	Cuml.
4	Gly(42)+AMP(36)	64	63	78	42	Cuml.
5	Gly(42)+ADP(41)	63	66	83	42	Cuml.
6	Ala(32)+Ser(39)	56	59	71	39	Cuml.
7	Ala(32)+Asp(51)	65	67	83	51	Cuml.
8	Ala(32)+AMP(36)	55	56	68	36	Cuml.
9	Ala(32)+ADP(41)	63	60	73	41	Cuml.
10	Ser(39)+Asp(51)	66	70	90	51	Cuml.
11	Ser(39)+AMP(36)	59	61	75	39	Cuml.
12	Ser(39)+ADP(41)	60	64	80	41	Cuml.
13	Asp(51)+AMP(36)	67	68	86	51	Cuml.
14	Asp(51)+ADP(41)	68	71	92	51	Cuml.
15	AMP(36)+ADP(41)	47	62	76	41	Ant.
16	Gly(42)+Ala(32)+Ser(39)	74	76	113		
17	Gly(42)+Ala(32)+Ser(39)+Asp(51)	78	88	164		
18	Gly(42)+Ala(32)+Ser(39)+Asp(51)+AMP(36)	80	92	200		
19	Gly(42)+Ala(32)+Ser(39)+Asp(51)+AMP(36)+ADP(41)	81	95	241		

[a]The biosynthetic activity was determine from the rate of Pi production.
[b]Values for cumulative inhibition were calculated by the procedure of Woolfolk and Stadtman (1967) according to the formula $X_i+(Y_i/100)\ (100-X_i)$, where X_i and Y_i are the values of percent inhibition observed with inhibitors X and Y alone, respectively.
[c]Ant.=Antagonistic inhibition, Cuml.=Cumulative inhibition.
[d]Abbreviations of inhibitors: Gly=glycine, Ala=L-alanine, Ser=L-serine, Asp=L-aspartic acid, AMP=adenosine 5′-phosphate, ADP=adenosine 5′-diphosphate. The concentration of inhibitors used was (in mM): Gly(25), Ser(25), Asp(25), AMP(5), ADP(5).
[e]Values in the parentheses are percent inhibition by individual inhibitior.

sions for partial noncompetitive inhibition (Segel, 1975):

$$\frac{1}{V_{max_i}}=\frac{1}{V_{max}}\cdot\frac{1+[I]/Ki}{1+\beta[I]/Ki}$$

and

$$\text{Slope}_i=\frac{Ks}{V_{max}}\cdot\frac{1+[I]/Ki}{1+\beta[I]/Ki}$$

Where Slope_i is the slope of the double reciprocal plot in the presence of a given inhibitor concentration, and β is the constant for the inhibitor to effect on the rate of decomposition of enzyme-substrate-inhibitor complex to product. The values of $\beta=0.602$ and $Ki=12.035$ mM were calculated from Fig. 4A when serine was given as the inhibitor; and the values of $\beta=0.271$ and $Ki=6.453$ mM were calculated from Fig. 4B when aspartic acid was given as the inhibitor. Replots of 1/V-axis intercept of reciprocal plots versus the concentrations of serine and aspartic acid were also hyperbolic as shown in the insets of Fig. 4. Double reciprocal plots of velocity versus ATP concentration in the range from

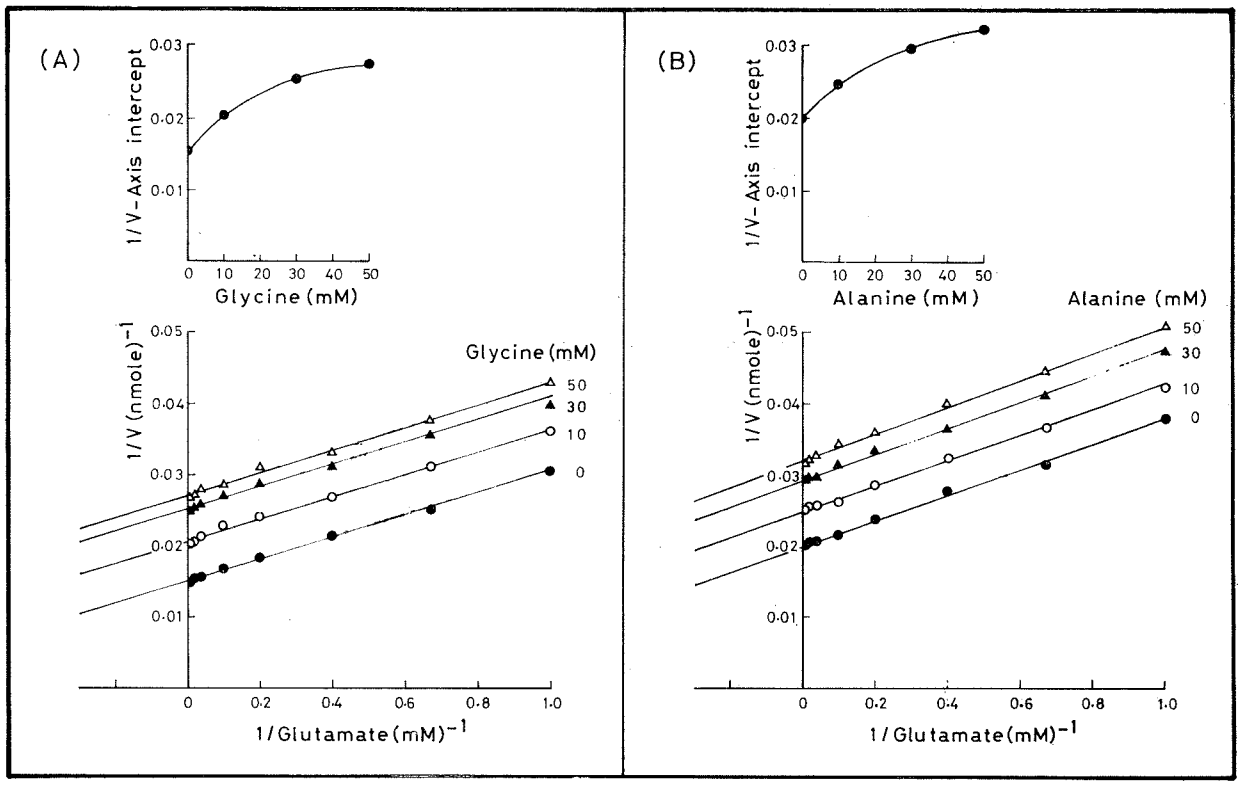

Fig. 3. Partial uncompetitive inhibition of rice leaf glutamine synthetase GSI by glycine and alanine. The biosynthetic activity was determined from the rate of Pi production. The reaction mixture contained 50 mM imidazole-HCl buffer (pH 7.0), 7.5 mM ATP, 50 mM NH_4Cl, 50 mM $MgCl_2$, enzyme and fixed concentrations of glycine or alanine. Glutamate concentration was varied in the range 1-100 mM. Double reciprocal plots of velocity versus glutamate concentration at the three indicated concentrations of glycine and alanine are shown in (A) and (B), respectively. (●) Minus glycine or alanine, (○) 10 mM glycine or alanine, (▲) 30 mM glycine or alanine, (△) 50 mM glycine or alanine. Insets: Replots of 1/V-axis intercept of reciprocal plots versus the concentrations of glycine and alanine are shown in (A) and (B), respectively.

0.25 to 7.50 mM at three concentrations of AMP and ADP are shown in Fig. 5. From the expressions for partial competitive inhibition (Segel, 1975):

$$-\frac{1}{Ks_{app}}=-\frac{1}{Ks}\cdot\frac{1+[I]/\alpha Ki}{1+[I]/Ki}$$

and

$$\text{Slope}_i=\frac{Ks}{V_{max}}\cdot\frac{1+[I]/Ki}{1+[I]/\alpha Ki}$$

The values of α=31.898 and Ki=1.418 mM were calculated from Fig. 5A when AMP was given as the inhibitor; and the values of α=15.924 and Ki=1.909 mM were calculated from Fig. 5B when ADP was given as the inhibitor. Replots of slope of reciprocal plots versus the concentrations of AMP and ADP were hyperbolic as shown in insets of Fig. 5.

Kinetics of inhibition of rice leaf glutamine synthetase GSII activity by glycine, alanine, serine, aspartic acid, AMP and ADP had also been studied. Since the affinity of GSII with respect to glutamate showed biphasic Km values at high and low glutamate concentrations (Guiz *et al.*, 1979; Ahmad *et al.*, 1982; Yuan and Hou, 1987), therefore, the inhibition of GSII activity by glycine, alanine, serine and aspartic

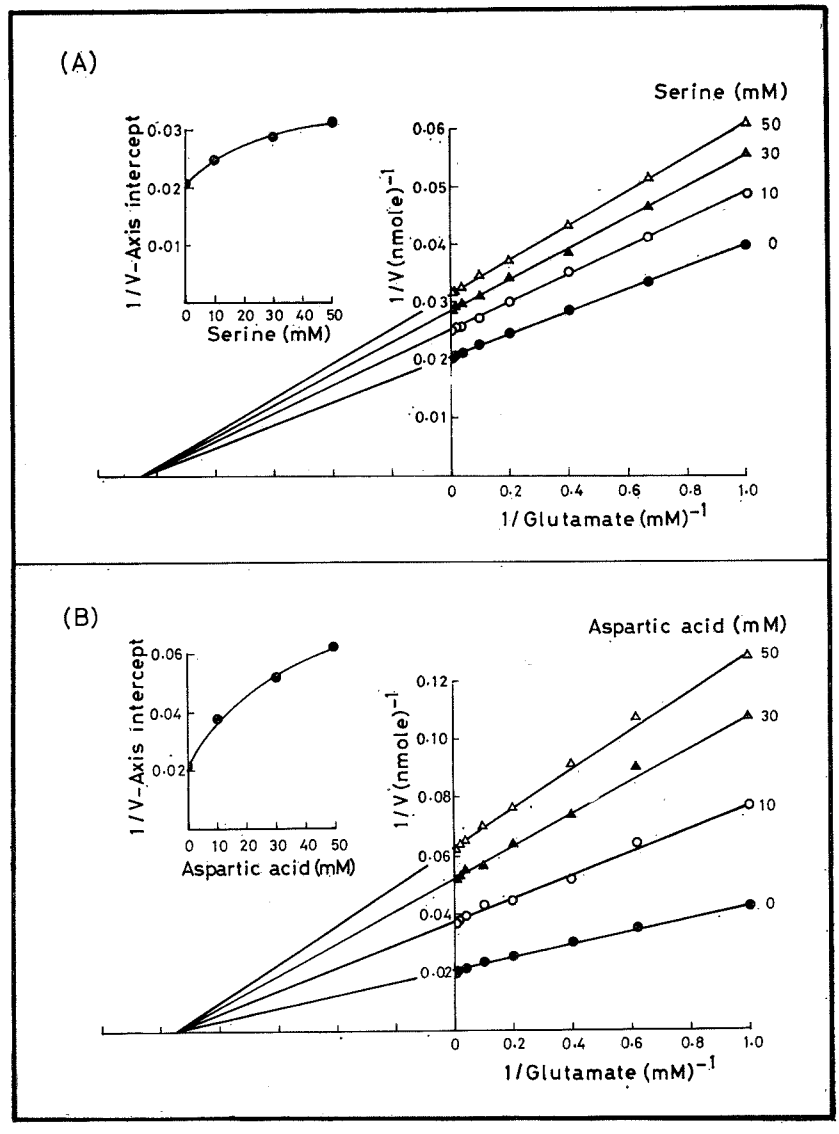

Fig. 4. Partial noncompetitive inhibition of rice leaf glutamine synthetase GS I by serine and aspartic acid. The biosynthetic activity was determined from the rate of Pi production. The reaction mixture contained 50 mM imidazole-HCl buffer (pH 7.0), 7.5 mM ATP, 50 mM NH_4Cl, 50 mM $MgCl_2$, enzyme and fixed concentrations of serine or aspartic acid. Glutamate concentration was varied in the range 1-100 mM. Double reciprocal plots of velocity versus the concentration of glutamate at the three indicated conentrations of serine and aspartic acid are shown in (A) and (B), respectively. (●) Minus serine or aspartic acid, (○) 10 mM serine or aspartic acid, (▲) 30 mM serine or aspartic acid, (△) 50 mM serine or aspartic acid. Insets: Replots of 1/V-axis intercept of reciprocal plots versus the concentration of serine and aspartic acid are shown in (A) and (B), respectively.

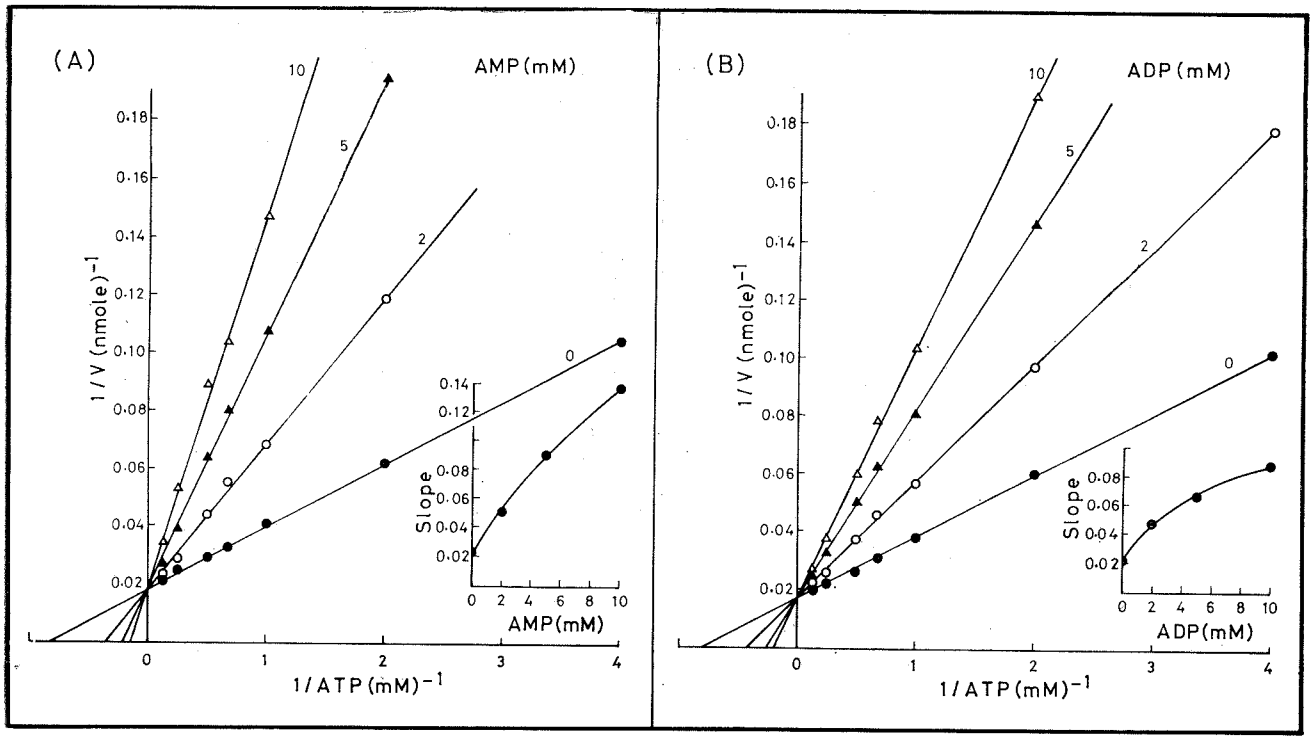

Fig. 5, Partial competitive inhibition of rice leaf glutamine synthetase GS I by AMP and ADP. The biosynthetic activity was determined from the rate of Pi production. The reaction mixture contained 50 mM imidazole-HCl bufer (pH 7.0), 100 mM glutamate, 50 mM NH_4Cl, 50 mM $MgCl_2$, enzyme and fixed concentrations of AMP or ADP. ATP concentration was varied in the range 0.25-7.50 mM. Double reciprocal plots of velocity versus ATP concentration at the three indicated concentrations of AMP and ADP are shown in (A) and (B), respectively. (●) Minus AMP or ADP, (○) 2 mM AMP or ADP, (▲) 5 mM AMP or ADP, (△) 10mM AMP or ADP. Insets: Replots of slope of reciprocal plots versus the concentration of AMP and ADP are shown in (A) and (B), respectively.

acid also showed biphasic inhibitory effects when glutamate concentration was varied in the range of 1 to 100 mM. At higher concentrations of glutamate (10-100 mM), glycine, alanine, serine and aspartic acid inhibited the GSII activity significantly, while at lower concentrations of glutamate (1-10 mM), the inhibitory effects of these amino acids on the GSII activity decreased markedly. At higher concentration of glutamate, partial mixed-type inhibition of GSII by glycine, alanine and aspartic acid, as well as partial noncompetitive inhibition by serine are depicted in Fig. 6. Double reciprocal plots of velocity versus the concentration of glutamate at three different concentrations of glycine, alanine, aspartic acid and serine are shown in Fig. 6. By using the expressions of partial mixed-type inhibition (Segel, 1975):

$$\frac{1}{V_{max_i}} = \frac{1}{V_{max}} \cdot \frac{1+[I]/\alpha Ki}{1+\beta[I]/\alpha Ki}$$

and

$$\frac{1}{V} \text{ at intersection} = \frac{1}{V_{max}} \cdot \frac{\alpha-1}{\alpha-\beta}$$

as well as

$$-\frac{1}{Ks_{app}} = -\frac{1}{Ks} \cdot \frac{1+[I]/\alpha Ki}{1+[I]/Ki}$$

and

$$-\frac{1}{Ks_{app}} \text{ at intersection} = -\frac{1}{Ks} \cdot \frac{1-\beta}{\alpha-\beta}$$

Where V is the initial velocity. The values of $\alpha=2.456$, $\beta=0.451$ and $Ki=3.209$ mM were calculated from Fig. 6A when glycine was given as

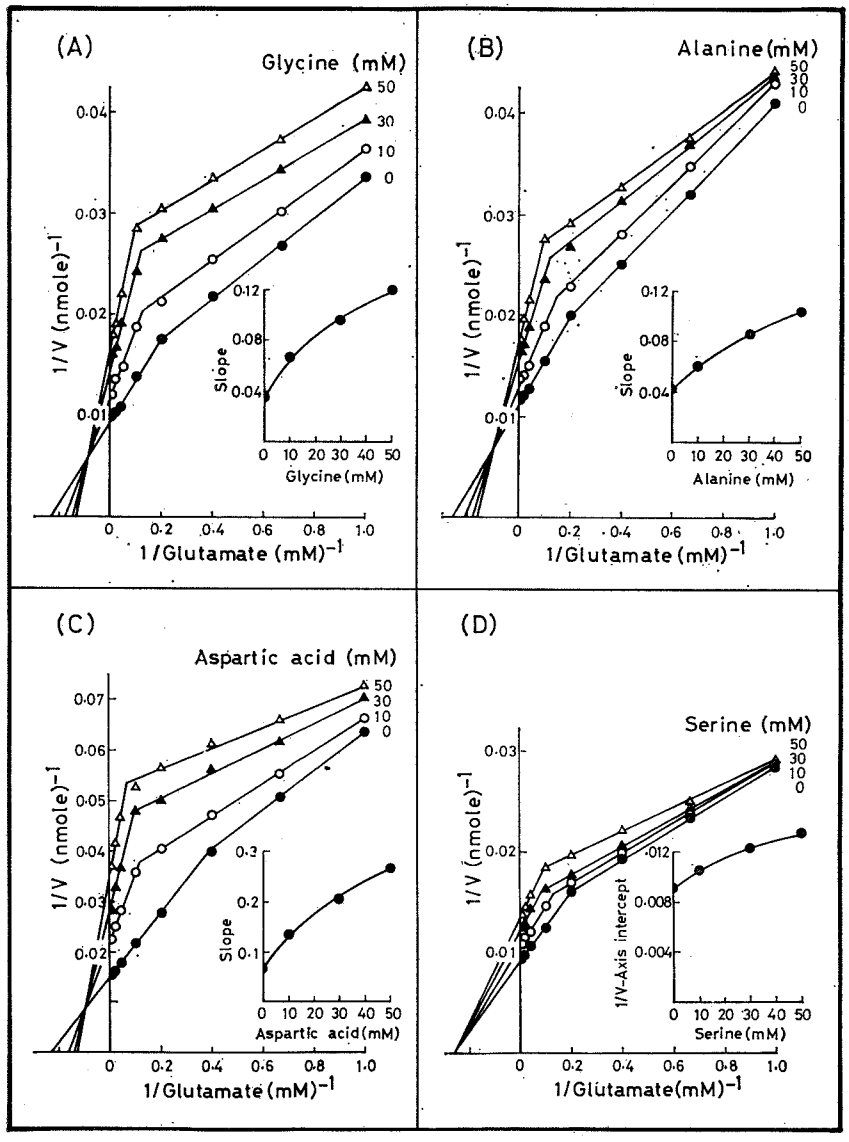

Fig. 6. Partial mixed-type inhibition of rice leaf glutamine synthetase GS II by glycine, alanine, aspartic acid, and partial noncompetitive inhibition by serine in the presence of higher glutamate concentration. The biosynthetic activity was determined from the rate of Pi production. The reaction mixture contained 50 mM imidazole-HCl buffer (pH 7.0), 7.5 mM ATP, 50 mM NH_4Cl, 50 mM $MgCl_2$, enzyme and fixed concentrations of glycine or alanine or aspartic acid or serine. Glutamate concentration was varied in the range 1-100 mM. Double reciprocal plots of velocity versus the concentration of glutamate at the three indicated concentrations of glycine, alanine, aspartic acid and serine are shown in (A), (B), (C) and(D), respectively. (●) Minus glycine or alanine or aspartic acid or serine, (○) 10 mM glycine or alanine or aspartic acid or serine, (▲) 30 mM glycine or alanine or aspartic acid or serine, (△) 50 mM glycine or alanine or aspartic acid or serine. Insets: Replots of slope of reciprocal plots versus the concentration of glycine, alanine and aspartic acid are shown in (A), (B) and (C), respectively; and replot of 1/V-axis intercept of reciprocal plots versus the concentration of serine is shown in (D).

the inhibitor; the values of α=1.985, β=0.348 and Ki=20.480 mM were calculated from Fig. 6B when alanine was given as the inhibitor; and the values of α=1.824, β=0.377 and Ki=6.219 mM were calculated from Fig. 6C when aspartic acid was the inhibitor. Replots of slope of reciprocal plots versus the concentrations of glycine, alanine and aspartic acid were hyperbolic as shown in insets of A, B and C of Fig. 6, respectively. Furthermore, according to the expressions for partial noncompetitive inhibition, the values of β=0.493 and Ki=29.995 mM were calculated from Fig. 6D when serine were given as the inhibitor. Replot of 1/V-axis intercept of reciprocal plots versus the concentration of serine was hyperbolic (inset in Fig. 6D). Partial competitive inhibition of GSII by AMP and ADP is depicted in Fig. 7. Double reciprocal plots of velocity versus ATP concentration in the range of 0.75 to 7.50 mM at three concentrations of AMP and ADP are shown in Fig. 7A and 7B, respectively. According to the expressions of partial competitive inhibition, the values of α=2.264 and Ki=3.684 mM were calculated from Fig. 7A with AMP as the inhibitor; and the values of α=3.545 and Ki=3.304 mM were calculated from Fig. 7B when ADP was the inhibitor. Replots of slope of reciprocal plots versus the concentrations of AMP and ADP were hyperbolic as shown in insets of Fig. 7. All values of Ki, α, and β for these six inhibitors are shown in Table 5.

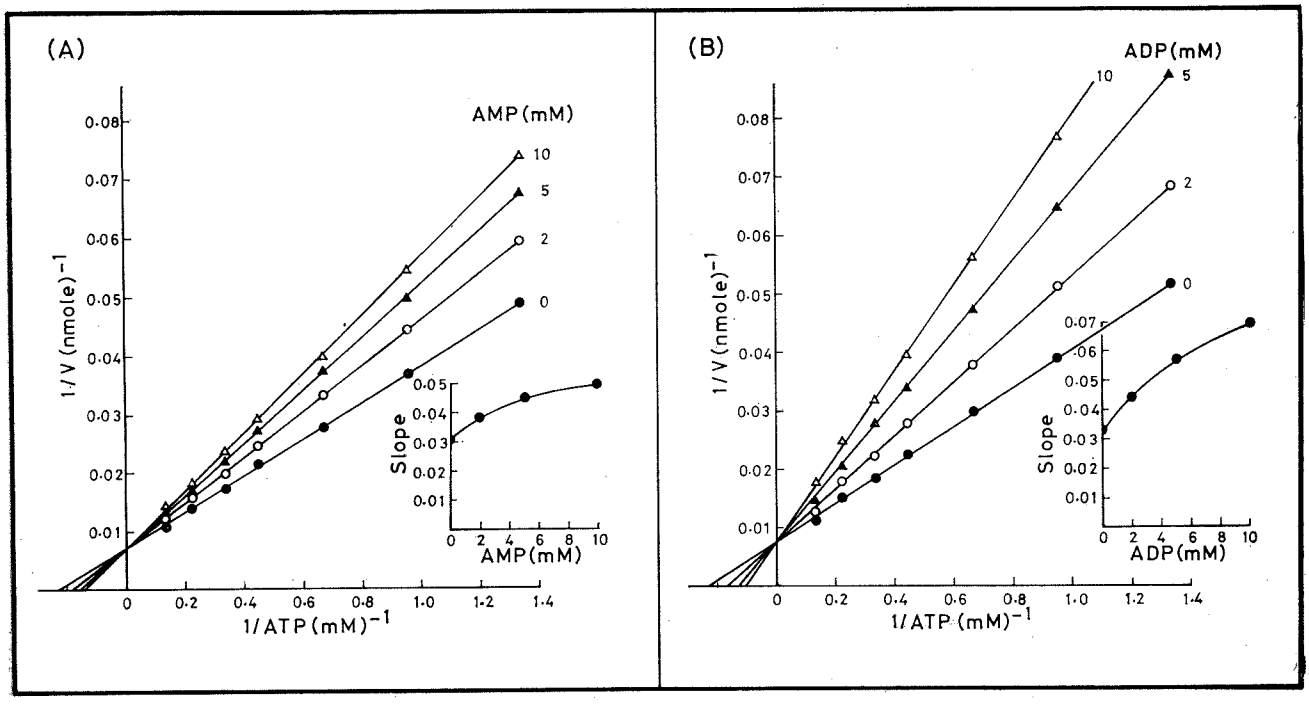

Fig. 7. Partial competitive inhibition of rice leaf glutamine synthetase GSII by AMP and ADP. The biosynthetic activity was determined from the rate of Pi production. The reaction mixture contained 50 mM imidazole-HCl bufer (pH 7.0), 100 mM glutamate, 50 mM NH_4Cl, 50 mM $MgCl_2$, enzyme and fixed concentrations of AMP or ADP. ATP concentration was varied in the range 0.75–7.50 mM. Double reciprocal plots of velocity versus ATP concentration at the three indicated concentrations of AMP and ADP are shown in (A) and (B), respectively. (●) Minus AMP or ADP, (○) 2 mM AMP or ADP, (▲) 5 mM AMP or ADP, (△) 10mM AMP or ADP. Insets: Replots of slope of reciprocal plots versus the concentrations of AMP and ADP are shown in (A) and (B), respectively.

Table 5. *Kinetic constants for the interaction of amino acids and nucleotides with the rice glutamine synthetases GSI and GSII*

Inhibitor	Type of inhibition*		*Ki* (mM)		α		β	
	GSI	GSII	GSI	GSII	GSI	GSII	GSI	GSII
Glycine	PUC	PMT	21.563	3.209	0.463	2.456	...	0.451
Alanine	PUC	PMT	25.480	20.480	0.534	1.985	...	0.348
Serine	PNC	PNC	12.035	29.995	...	...	0.602	0.493
Aspartic acid	PNC	PMT	6.453	6.220	...	1.824	0.271	0.377
AMP	PC	PC	1.418	3.684	31.898	2.264	...	...
ADP	PC	PC	1.909	3.304	15.924	3.545	...	...

*PUC, partial uncompetitive; PMT, partial mixed-type; PNC, partial noncompetitive; PC, partial competitive.

Discussion

Attention was focused upon glutamine synthetase as a central target for cellular regulation of glutamine metabolism. In order to examine whether rice leaf glutamine synthetases GSI and GSII are susceptible to feedback control by products of glutamine metabolism, the ability of various amino acids and nucleotides to inhibit these enzymes were tested. The results presented in tables 1 and 2 showed that the activities of GSI and GSII were significantly inhibited by glycine, alanine, aspartic acid, serine, AMP and ADP. The responses of glutamine synthetases GSI and GSII to increasing concentrations of each of these six most potent inhibitors are shown in Figs. 1 and 2. A notable characteristic of all these inhibitors is their failure to achieve complete inhibition at essentially saturating concentrations. Although the extent of inhibition varied with different inhibitors, it never exceeded 50% in inhibition under the assay conditions used in this study. However, the responses of GSII to increasing concentrations of each of these four amino acids were less sensitive than that of GSI. Furthermore, the ordinate intercept values for these six inhibitors shown in insets of Figs. 1 and 2 were greater than unity, indicating that the activities of GSI and GSII are partially inhibited by these inhibitors (Welder *et al.*, 1976).

The regulation of enzyme activity by partial inhibition of end products was reported for several enzymes (Woolfolk and Stadtman, 1967; Wang *et al.*, 1970; Gold *et al.*, 1974; Rhee *et al.*, 1977), and a number of hypotheses have been proposed to explain the partial inhibition (Segel, 1975; Woolfolk and Stadtman, 1967; Rhee *et al.*, 1977). According to Woolfolk and Stadtman (1967), the partial inhibitory effects could be explained by at least four situations: (a) The existence of a single enzyme with a single non-specific allosteric site. Binding to this site by any one of inhibitors would cause a conformational change resulting in a catalytically less active form of the enzyme. In this situation, the total inhibition in the presence of more than one inhibitor will be no more than that obtained by the saturating level of the most effective inhibitor of the mixture. (b) The existence of a heterogenous population of closely related isoenzymes that differ from each other only in their specific susceptibility to different inhibitors. (c) The existence of a single enzyme possessing multiple catalytic sites that differ from each other in the specificity of their susceptibility to different inhibitors.

In the situation (b) or (c), the total inhibition produced by a mixture of inhibitors will be equal to the sum of the inhibitory effects caused by each inhibitor when tested individually, thus the inhibitory effects will be additive when more than one inhibitor are present at saturating concentrations. (d) The existence of a single enzyme possessing separate inhibitor sites each of which is specific for only one of different inhibitors. In this situation, the total inhibition caused by a mixture of inhibitors could be either greater or less than the sum of the inhibitory effects caused by each inhibitor when tested individually. Greater inhibition would result if different inhibitors are synergistic in their action; whereas less than additive inhibition would result if there is antagonism between different inhibitors, or if each of these inhibitors is completely independent of its action on the enzyme. If two inhibitors act on a single enzyme, antagonism occurs when the second inhibitor produces less effect in the presence of the first inhibitor than it does alone, and synergism occurs when the effect is greater. If these different inhibitors are completely independent of their action, the activity of the enzyme is progressively decreased by increasing the number of inhibitors; therefore, the combined inhibitory effects caused by a mixture of inhibitors will be cumulative inhibition (Woolfolk and Stadtman, 1967).

To determine which of the above inhibition patterns is exhibited by rice leaf glutamine synthetases GSI and GSII, the total inhibition produced by various combinations of inhibitors were examined. The results indicate that the above listed situations (a), (b) and (c) could be ruled out in the cases of GSI and GSII. The results in tables 3 and 4 showed that all values of the observed inhibition by pairs of inhibitors are greater than that observed for either one of the two when tested individually, and is also much less than the sum of the inhibitions obtained by the individual inhibitors when tested separately. In this case, it is not only inconsistent with the occurrence of a common binding site for all inhibitors, but also disagreeable with the occurrence of inhibitor specific isoenzymes or the differential inhibition of multiple catalytic sites on a single enzyme. On the other hand, the value of observed inhibition caused by each pair of inhibitors is greater than the observed inhibition caused by the most potent inhibitor of this pair and much less than the sum of the inhibitory effects caused by each inhibitor when tested individually. This case is consistent with the situation (d) mentioned above. For judging the inhibition patterns, we consider that if the observed inhibition caused by the pair of inhibitors is near by the observed inhibition caused by the most potent inhibitor of this pair, it belongs to antagonistic inhibition; if the observed inhibition caused by the pair of inhibitors is equal to or near by the calculated value for cumulative mechanism, it belongs to cumulative inhibition. According to the above description, the combined inhibitory effect of each pair of inhibitors on the activity of GSI (experiments 1 to 15 in Table 3.) show that 6 pairs are cumulative inhibition and 9 pairs are antagonistic inhibition; however, the inhibitory effect on the activity of GSII (experiments 1 to 15 in Table 4) show that 14 pairs are cumulative inhibition and only one pair is antagonistic inhibition. The antagonistic inhibition occurred in many pairs of inhibitors on the activity of GSI is further identified by combinations of more than two inhibitors. The data of experiments from 16 to 19 in Table 3 show that as the number of inhibitors increased, the value of observed inhibition increases but is much less than the calculated value for cumulative mechanism. However, the cumulative inhibition occurred in the most pairs of inhibitors on the

activity of GSII is also further identified by combinations of more than two inhibitors. The data of experiments from 16 to 19 in Table 4 show that as the number of inhibitors increased, the extent of observed inhibition increases in a cumulative manner.

As to various antagonistic inhibitor-inhibitor interactions noted in tables 3 and 4, there are at least two different explanations (Welder *et al.*, 1976). One is direct overlap or competition between inhibitors for a common binding domain. The other explanation involves spatially separate sites that interact antagonistically. However, the present data do not serve to distinguish between these two models. If one assumes that the direct overlap model predominates for the spatial relations among inhibitor sites, one can visualize a very compact arrangement of the sites. Alternatively, if one assumes that the separate site model is being true in the most cases, the logical consequence is that the enzyme maintains as many binding sites for inhibitors as its counterparts. Therefore, the results shown in tables 3 and 4 indicate that the rice leaf glutamine synthetases GSI and GSII should possess separate inhibition sites for each of these six inhibitors. Furthermore, the results also indicate that the arrangement of these inhibition sites on the sulface of GSI molecule is more compact than that on GSII.

The occurrence of separate binding sites for different inhibitors on GSI and GSII is further supported by the kinetics of inhibition. The kinetics of inhibition of GSI atcivity by glycine, alanine, aspartic acid, serine, AMP and ADP fall into three categories. With respect to glutamate, glycine and alanine are partial uncompetitive inhibitors (Fig. 3), aspartic acid and serine are partial noncompetitive inhibitors (Fig. 4). With respect to ATP, AMP and ADP are partial competitive inhibitors (Fig. 5). Replots of 1/V-axis intercept of the primary reciprocal plots versus the concentration of each of glycine, alanine, aspartic acids and serine (insets in Figs. 3 and 4) as well as replots of slope of the primary reciprocal plots versus the concentration of AMP and ADP (insets in Fig. 5) are hyperbolic further supporting for partial inhibition (Segel, 1975). The kinetics of inhibition of GSII by these six inhibitors also fall into three categories: With respect to glutamate at higher concentration, glycine, alanine and aspartic acid are partial mixed-type inhibitors; and serine is partial noncompetitive inhibitor (Fig. 6). With respect to ATP, AMP and ADP are partial competitive inhibitors (Fig. 7). Replots of slope of the primary reciprocal plots versus the concentration of each of glycine, alanine, aspartic acid, AMP and ADP (inset in Figs. 6 and 7) as well as replots of 1/V-axis intercept of the primary reciprocal plots versus the concentration of serine (inset in Fig. 6) are hyperbolic also indicating a further evidence for partial inhibition (Segel, 1975).

The partial inhibition observed with rice leaf glutamine synthetases GSI and GSII could be fitted into the model of Stadtman *et al.* (1968) and Segel (1975). In the model of Stadtman *et al.* (1968), each inhibitor possesses two distinct and mutually exclusive binding sites, the inhibitory site and the noninhibitory site. When the inhibitor is bound to the inhibitory site, it induces a conformational change in the enzyme that either prevents the substrate from binding or decreases the catalytic efficiency even though the substrate is bound to the enzyme. When the inhibitor is bound to the noninhibitory site, binding to inhibitory site is prevented without any effect on the catalytic efficiency and on the binding of substrate. Although the data of partial inhibition could be explained by the two-site model, an alternative explanation based on the model of Segel (1975) could be more suitable for the present data. In the model of Segel

(1975), the inhibitors bind to their single specific sites to yield enzyme-inhibitor and enzyme-inhibitor-substrate complexes. The enzyme-inhibitor-substrate complex could yield products with equal or less facility than the enzyme-substrate complex. The inhibitors exert their action either by increasing the apparent Km value or decreasing the reaction velocity V of the enzyme. The partial inhibition caused by any inhibitor, I, can be represented generally by the following scheme:

$$\begin{array}{ccccc} E + S & \overset{Ks}{\rightleftharpoons} & ES & \overset{k_p}{\longrightarrow} & E + P \\ + & & + & & \\ I & & I & & \\ Ki \Big\| & & \alpha Ki \Big\| & & \\ EI + S & \underset{\alpha Ks}{\rightleftharpoons} & EIS & \underset{\beta k_p}{\longrightarrow} & EI + P \end{array}$$

The alternation of Ks due to bound inhibitor is expressed by an interaction constant α, the inhibitor effect on the rate of decomposition of the EIS complex to product is denoted by the constant β, and k_p is the rate constant for the breakdown of ES to product. According to the description of Segel (1975) for the partial inhibition, the type of inhibition can be predicted from the scheme depending on the values of α and β. when $1<\alpha<\infty$ and $\beta=1$, the bound inhibitor only partially blocks binding substrate and does not alter the rate of product formation from substrate, then we have partial competitive inhibition. The α and Ki values for AMP and ADP in Table 5 indicate that the partial competitive inhibition by AMP and ADP on GSI and GSII are consistent with this case. When $\alpha=1$ and $0<\beta<1$, substrate binding is not affected, but product formation from EIS is slower than from ES, then we have partial noncompetitive inhibition. The β and Ki values for serine and aspartic acid in Table 5 indicate that the partial noncompetitive inhibition by serine and aspartic acid on GSI as well as the partial noncompetitive inhibition by serine on GSII are consistent with this case. When $0<\alpha<1$, $0<\beta<1$ and $\alpha=\beta$, binding to free enzyme is not affected, but the rate of product formation is decreased by the presence of inhibitor, then we have uncompetitive inhibition. The α and Ki values for glycine and alanine in Table 5 indicate that the partial uncompetitive inhibition by glycine and alanine on GSI are consistent with this case. When $1<\alpha<\infty$ and $0<\beta<1$, both E and EI bind to substrate but EI is with lower affinity, and both ES and EIS form products, but EIS is with less productivity, then we have partial mixed-type inhibition. The α, β and Ki values for glycine, alanine and aspartic acid in Table 5 indicate that the partial mixed-type inhibition by glycine, alanine and aspartic acid on GSII are consistent with this case. This mixed-type system may be considered to be a mixture of partial competitive and partial noncompetitive inhibition.

In conclusion, these various lines of evidence appear to be the most compatible with the existence of rice leaf glutamine synthetases GSI and GSII with separate inhibition sites for each of these six inhibitors. However, it is very difficult to imagine a mechanism that achieves partiality of the inhibitory responses at saturating concentrations of each inhibitor, and it is also difficult to understand how six separate inhibitors could react with a single enzyme molecule at different binding sites to produce predictable conformational changes. Glutamine synthetase occupies a key position in a highly branched nitrogen metabolic pathway, and its activity is under rigorous cellular control. The partial inhibition of rice leaf glutamine synthetases GSI and GSII demonstrated here by glycine, alanine, serine, aspartic acid, AMP and ADP might be a part of the physiological regulatory mechanism of nitrogen metabolism in rice plants. Nevertheless, all of the evidence presented here is indirect, therefore the final decision as to the

nature and number of binding sites must await more conclusive studies in which binding of the various inhibitiors is measured directly.

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胺基酸與核苷酸對於水稻葉片中麩醯胺合成酶活性之調節特性

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使用各種胺基酸和核苷酸作爲抑制劑，分別測試其對於水稻葉片中麩醯胺合成酶 GSⅠ 與 GSⅡ 之由鎂離子支持的合成活性的抑制作用。發現其中最具抑制能力者計爲甘胺酸 (glycine)、丙氨酸 (alanine)、天冬氨酸 (aspartic acid)、絲氨酸 (serine)、腺苷一磷酸 (AMP) 及腺苷二磷酸 (ADP) 等六種。當這六種抑制劑之濃度分別增加至飽和程度時，則 GSⅠ 與 GSⅡ 之殘餘百分活性皆隨着降低，而且都趨向一有限值。此現象顯示該六種抑制劑之抑制作用系屬於部份抑制作用 (partial inhibition)，同時由分數抑制的分析和抑制動力學的數據，亦證實了此一部分抑制作用。將此六種抑制劑以配對方式組合成15對。各配對對於 GSⅠ 活性之聯合抑制作用爲6對屬於蓄積性抑制 (Cumulative inhibition)，而9對屬於拮抗性抑制 (antigonistic inhibition)；可是各配對對於 GSⅡ 活性之聯合抑制作用爲14對屬於蓄積性抑制，而僅一對爲拮抗性抑制。此結果顯示 GSⅠ 與 GSⅡ 皆具有許多分離的結合部位，分別適用於此六種抑制劑，但是這些抑制部位在這兩種酶的表面上，部位間產生的交互作用不一樣。若以麩胺酸 (glutamate) 爲變動基質，甘胺酸和丙氨酸爲 GSⅠ 之部分偶聯性 (partial uncompetitive) 抑制劑，而絲氨酸和天冬氨酸則爲部分非競爭性 (partial noncompetitive) 抑制劑；可是甘胺酸、丙氨酸和天冬氨酸爲 GSⅡ 之部分混合型性 (partial mixed-type) 抑制劑，而絲氨酸則爲非競爭性抑制劑。若以腺苷三磷酸 (ATP) 爲變動基質，則腺苷一磷酸和腺苷二磷酸皆爲 GSⅠ 與 GSⅡ 之部分競爭性 (partial competitive) 抑制劑。