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Studies on glutamine synthetase. Purification of the enzyme from mung bean (*Phaseolus aureus*) seedlings and modulation of the enzyme–antibody reaction by the substrates

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Abstract. Glutamine synthetase (L-glutamate : ammonia ligase, EC 6.3.1.2) from *Phaseolus aureus* (mung bean) seedlings was purified to homogeneity by ammonium sulphate fractionation, DEAE-cellulose chromatography, Sephadex G-200 gel filtration and affinity chromatography on histidine-Sepharose. The enzyme had a molecular weight of 775,000 ± 25,000. The enzyme consisted of identical subunits with an approximate subunit molecular weight of 50,000. Hyperbolic saturation curves were obtained with the substrates, glutamate, ATP and hydroxylamine.

Antibody, raised in the rabbit, against mung bean glutamine synthetase, completely inhibited the activity of the enzyme. Preincubation of the enzyme with glutamate and ATP, prior to the addition of the antibody, partially protected the enzyme against inhibition. The K_m values of this enzyme-antibody complex and the native enzyme were identical (glutamate, 2.5mM; ATP, 1 mM; hydroxylamine, 0·5 mM). The Km values of the partially inhibited enzyme (the enzyme pretreated with antibody prior to the addition of substrates) were 2-fold higher than those of the native enzyme. These results suggested that the substrate-induced conformational changes in the enzyme were responsible for the protection against inhibition of the enzyme activity by the antibody.

Keywords. Mung bean; glutamine synthetase; antibody interactions; *Phaseolus aureus*.

Introduction

Earlier results from this laboratory (Seethalakshmi, 1979) showed that the activity of mung bean glutamine synthetase was regulated by the amino acid and nucleotide end products of glutamine metabolism, in a manner similar to that reported in *Escherchia coli* by Stadtman (1973). The γ-glutamyl transferase reaction catalysed by this enzyme proceeded by a ping pong mechanism (Seethalakshmi *et al.,* 1977). Glutamine synthetase, catalyses a *ter ter* biosynthetic reaction shown below, and

therefore, serves as an excellent model for understanding the substrate-induced conformational changes. In this study, the enzyme-antibody reaction has

$$\text{Glutamate} + \text{ATP} + \text{NH}_4+ \xrightarrow{Me^{2+}} \text{Glutamine} + \text{ADP} + \text{P}_4$$

been used to probe the conformational changes in the enzyme.

Materials and methods

Materials

The following chemicals were purchased from Sigma Chemical Company, St. Louis, MO, USA : imidazole, ß-mercaptoethanol, ATP, diethylaminoethyl (DEAE)-cellulose, sodium L-glutamate, hydroxylamine hydrochloride, EDTA, Coomassie brilliant blue R, sodium dodecyl0 sulphate, N, N′-methylene *bis* acrylamide, N, N, N′, N′-tetramethylene diamine (TEMED), riboflavin, tris (hydroxymethyl) aminomethane, bovine serum albumin, chymotrypsin, bovine liver glutamate hydrogenase, catalase, *Escherichia coli* ß-galactosidase, l-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, hexamethylenediamine, ferritin and the amino acids. Sepharose-4B, Sephadex G-200 and Sephadex G-25 were purchased from Pharmacia, Uppsala, Sweden. Agar and Freund's complete adjuvant were obtained from Difco Lab., Detroit, MC, USA. All other reagents used in this investigation were of the analytical reagent grade Mung bean seeds were purchased from the local market.

Methods

Enzyme assay: Glutamine synthetase activity was measured by estimating the γ-glutamylhydroxamate formed in the reaction mixture (Rowe *et al.,* 1970). The reaction mixture (1·0 ml) contained the following components : 80 mM imidazole hydrochloride buffer (pH 7·2), 10 mM MgCl2, 10 mM ß-mercaptoethanol 100 mM glutamate, 10 mM ATP, 10 mM hydroxylamine (freshly neutralised to pH 7 ·2) and an appropriate amount of the enzyme. After incubation at 37° C for 15min, the reaction was stopped by the addition of 1·5 ml of ferric chloride reagent. The precipitated proteins were removed by centrifugation. The absorbance of the supernatant solution was measured at 535 nm in a Pye Unicam SP-500 spectrophotometer.

Preparation of histidine-Sepharose: Aminohexane-Sepharose (20 ml), prepared as described by March *et al.* (1974), was washed thoroughly with glass-distilled water. Histidine hydrochloride (2 mmol, pH 4·5) was added to the gel and the mixture was gently stirred. The carboxylate moiety of histidine was coupled to the amino group of the gel by the addition of 0·1 M carbodiimide and the mixture was gently stirred for 24 h at 4° C. This preparation was then washed with water and stored at 4°C.

Protein estimation: Protein content was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as the standard,

Disc gel electrophoresis: Analytical polyacrylamide gel electrophoresis in 5% gel was performed as described by Davis (1964). Electrophoresis of the sodium dodecyl sulphate dissociated proteins was carried out as described by Weber and Osborn (1969).

Double diffusion test: The antibody to the enzyme was raised by repeated injection of mung bean glutamine synthetase (step 6, table 1) into a male rabbit. Antiserum was obtained by centrifuging the blood collected by puncturing the marginal ear vein. The γ-globulin fraction isolated by ammonium sulphate fractionation of the antiserum was used in this study. The Ouchterlony plates were prepared with 1·5% agar in 0·01 M sodium phosphate buffer (pH 7·5) containing 0·14 M sodium chloride and 0·02% sodium azide. After placing the antigen and antibody in the wells cut into agar plates, the plates were developed in a humid chamber at 0–5° C for 24 h.

Purification of the enzyme: All operations were carried out at 0–4° C and all centrifugations at 12,000 g for 10 min in a Sorvall RC–2B centrifuge.

Mung bean seedlings (800 g; germinated for 24 h at 37° C) were washed and homogenised for 2 min at 30 sec intervals in 500 ml of 0·1 M imidazole hydrochloride buffer (pH 7·2) in a precooled Waring blendor. The homogenate was squeezed through cheese-cloth and centrifuged. The supernatant solution was designate as the crude extract(step 1, table 1). Protamine sulphate (2% solution) was added with constant stirring to the crude extract such that the protamine sulphate to protein ratio was 1:5 After 15 min, the precipitated nucleoproteins were removed by centrifugation (step 2) Solid ammonium sulphate was added to the supernatant solution to obtain 0·45 of saturation. After 10 min, the precipitate was removed by centrifugation. The supernatant fraction was raised to 0·6 of saturation by a further addition of solid ammonium sulphate and the precipitate was dissolved in a small volume of 0·02 M imidazole hydrochloride buffer

Table 1. Purification of glutamine synthetase from the germinated seedlings of *Phaseolus aureus* (mung bean).

Step	Fraction	Total protein (mg)	Total units*	Specific activity	Fold-purification	Recovery %
1	Crude	13,714	192	0·014	..	100
2	Protamine sulphate	5,636	248	0·044	3	130
3	Ammonium sulphate	989	184	0·186	13	95
4	Alumina-Cγ gel	418	128	0·306	22	67
5	DEAE-cellulose	44	47	1·08	77	24
6	Sephadex G–200	1·4	8	5·6	400	4
7	Affinity chromatography	0·4	6	15	1070	3

*μmol γ-glutamylhydroxamate formed/min.

(pH 7·2) and desalted by passage through a Sephadex G-25 column (1·5 × 80 cm) previously equilibrated with 0·02 M imidazole hydrochloride buffer (pH 7·2) (step 3). Alumina-Cγ gel was prepared as described by Willstatter and Kraut (1923). The enzyme fraction was added to alumina Cγ gel (0·4mg of gel per mg of protein) and gently stirred for 15 min. The supernatant solution obtained on centrifugation was designated as alumina-Cγ gel supernatant (step 4) The enzyme fraction (420 mg) was loaded onto a DEAE-cellulose column (1·4 × 65 cm) equilibrated with 0·02 M imidazole hydrochloride buffer (pH 7·2) containing 10mM β-mercaptoethanol, 0·l mM EDTA, 0·1 mM ADP and 0·075 M sodium chloride. The column was washed with the same buffer until the absorbance of the eluate at 280 nm was reduced to 0·05. A linear sodium chloride gradient (0·075-0·2 M) with a total volume of 500 ml was applied-Fractions (2 ml) were collected, and assayed for enzyme activity. The enzyme was eluted between 0·15–0·19 M sodium chloride. The active fractions were pooled and the enzyme was precipitated by the addition of solid ammonium sulphate to 0·60 of saturation. The precipitate was dissolved in 0·02 M imidazole hydrochloride buffer (pH 7·2) containing 10 mM β-mercaptoethanol, 0·l mM EDTA, 0·1 mM ADP and 0·1 M sodium chloride (step 5).

The enzyme obtained from the previous step was applied on a Sephadex G-200 column (1·4 × 65 cm) which had been previously equilibrated with 0·02 M imidazole hydrochloride buffer (pH 7·2) containing 10 mM β-mercaptoethanol, 0·1 mM EDTA, 0·l mM ADP and 0·1 M sodium chloride. The enzyme was eluted with the same buffer. Fractions (1ml) were collected and assayed for enzyme activity. The enzyme activity appeared immediately after the void volume. The fractions with high specific activity (> 4·5) were pooled and used for further purification (step 6).

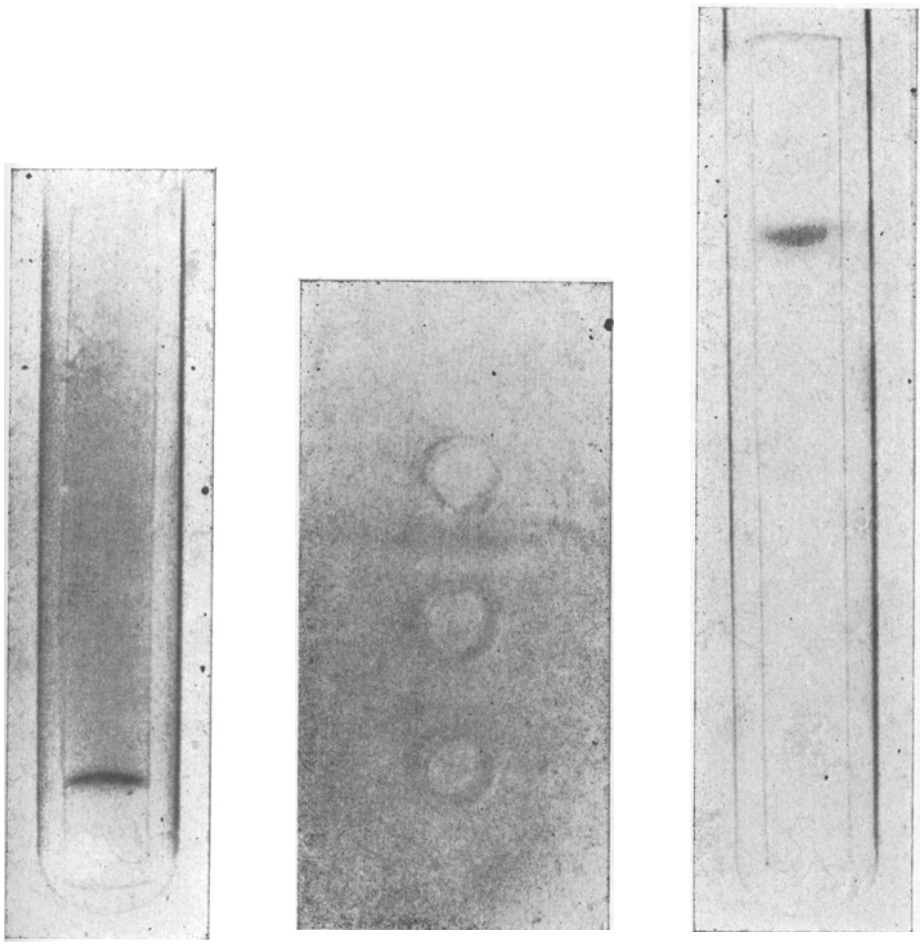
Histidine-Sepharose was packed into a column (1×10 cm) and equilibrated with 0·02 M imidazole hydrochloride buffer (pH 7·2) containing 10 mM β-mercaptoethanol, 0·1 mM ADP, 0·1 mM EDTA and 0·05 M sodium chloride. The enzyme fraction from step 6 was diluted with the buffer to reduce the sodium chloride concentration to 0·05 M and loaded onto the column. The column was washed with the equilibrating buffer till the absorbance of the eluants was less than 0·05. The enzyme was eluted from the column with the same buffer containing 50 mM glutamate, 2 mM ATP and 2 mM magnesium chloride. Fractions (1ml) were collected, assayed for enzyme activity and pooled. A summary of the purification procedure is given in table 1, This procedure resulted in a 1000-fold purification with 3% recovery of the enzyme activity.

One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmol of γ-glutamylhydroxamate per min at pH 7·2 and 37° C.

Results

Criteria of purity

The purified enzyme gave a single band when subjected to electrophoresis in 5% gel in Tris-glycine buffer (pH 8·6) (figure 1). The purity of the enzyme preparation was confirmed by the Ouchterlony double diffusion test. Figure 2 shows a single antigen-antibody precipitin band. Two faint bands, in addition to the



(1) **(2)** **(3)**

Figure 1. Polyacrylamide gel electrophoresis of mung bean glutamine synthetase Electrophoresis was carried out in 5% acrylamide gel in 0·1 M Tris-glycine buffer (pH 8·6) for 1h. The migration of the protein (50 μg) was from top (cathode) to the bottom (anode). A single band was obtained even when 100 μg of the protein was used.

Figure 2. Ouchterlony double diffusion analysis of mung bean glutamine synthetase against its anitbody.
The centre well contained the y−globulin fraction of the rabbit antiserum. Well11: enzyme obtained from step 6 (table 1). Well 2: enzyme obtained from step 7 (table 1).

Figure 3. Sodium dodecyl sulphate gel electrophoresis of mung bean glutamine synthetase.

Mung bean glutamine synthetase (50 μg) was subjected to denaturation in the presence of sodium dodecyl sulphate and ß-mercaptoethanol and the electrophoresis was carried out according to the method of Weber and Osborn (1969).

major precipitin band, were observed when the enzyme from step 6 was used. These bands are not clear in the photographs.

Molecular weight

The molecular weight of the enzyme was determined by gel filtration on Sepharose-4B. The marker proteins used in this analysis were *ß*-galactosidase (520,000), ferritin (480,000), catalase (240,000) and glutamate dehydrogenase (350,000). From the standard curve (not shown), the molecular weight of mung bean glutamine synthetase was calculated to be 775,000 ± 25,000.

Subunits

The subunit composition of the enzyme was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Bovine serum albumin, cytochrome c, ovalbumin, chymotrypsin were used as the marker proteins. Figure 3 depicts the migration of the single subunit of mung bean glutamine synthetase. From the calibration curve (not shown), the molecular weight of the single subunit was found to be approximately 50,000

Properties of the enzyme

The enzyme reaction was linear with time upto 15 min and enzyme concentration upto 20*µg.* The enzyme was optimally active at pH 7·2 and 45° C. Hyperbolic saturation curves were obtained with glutamate, ATP and hydroxylamine. The K_m values for glutamate, hydroxylamine and ATP, calculated from the linear Line weaver-Burk plots, were 2·5, 0·5 and 1 mM, respectively (data not shown).

Inhibition of mung bean glutamine synthetase activity by its antibody

Figure 4a depicts the inhibition of mung bean glutamine synthetase activity on increasing the concentration of the antibody. Amounts of antibody greater than 200/µg almost completely inhibited the enzyme activity. A value of 0·01 was obtained for the intercept on the ã-axis in the plot of reciprocal of per cent inhibition against reciprocal of antibody concentration (inset figure 4), indicating complete inhibition. Values greater than 0·01 would indicate partial inhibition.

Protection of glutamine synthetase activity against inhibition by antibody

It can be seen from table 2 that glutamate in the presence of ATP affords partial protection against inhibition by antibody. However, these substrates when present alone in the preincubation medium were not effective in protecting the enzyme against inhibition. Hydroxylamine was not effective either when present alone or in combination with either ATP or glutamate. The enzyme pretreated with glutamate and ATP is designated as the protected, enzyme .

The protected enzyme was preincubated with varying amounts of antibody indicated in figure 4 (curve b). The maximum amount of inhibition of the protected enzyme was only 70%.

In view of the protection afforded by saturating concentrations of glutamate and ATP, it was of interest to determine the minimum concentration of glutamate and ATP required for protection. The results shown in tables 3 and 4 suggested that concentrations of ATP (0·05mM) and glutamate (2mM) less than their K_m values (1 and 2·5 mM, respectively) were able to maximally protect the enzyme against inhibition by the antibody. The protection could be due to a conformational

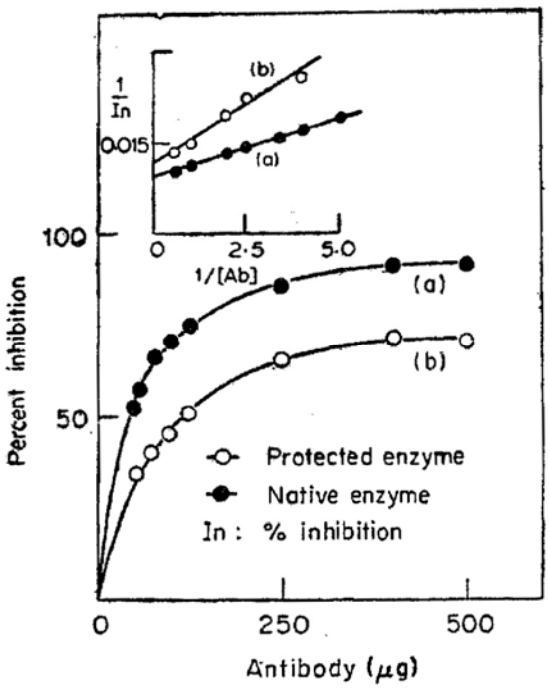


Figure 4. Effect of antibody on the activity of the native and the protected mung bean glutamine synthetase.
(a) The enzyme (5–6 μg) was preincubated with varying amounts of γ–globulin fraction for 5 min at 37° C and the reaction was started by the addition of the saturating concentrations of the substrates (glutamate, 100 mM; hydroxylamine, 10mM; ATP, 10mM). The velocity was determined by estimating the γ-glutamylhydroxamate formed in the reaction mixture, (b) The enzyme (5–6 μg) was preincubated with saturating concentrations of glutamate (100 mM) and ATP (10 mM) for 5 min at 37° C. This enzyme was designated as the protected enzyme. This was followed by the second pre-incubation with varying amounts of γ–globulin fraction for 5 min at 37° C. The reaction was started by the addition of saturating concentration of hydroxylamine (10 mM). The velocity was determined by estimating the γ-glutamylhydroxamate formed in the reaction mixture.
Inset: Double reciprocal plot of per cent inhibition of the enzyme activity versus the concentration ofantibody.
(– O – O –) Protected enzyme ; (–●–●–) Native enzyme. In: per cent inhibition.

change in the enzyme resulting in decreased ability of the antibody to interact with the enzyme or due to the possibility that antigen-antibody complex may be partially active. It was, therefore, of interest to determine the kinetic parameters of the three enzyme species, viz., native, protected and inhibited glutamine synthetase

Comparison of kinetic parameters of the native, protected and the inhibited glutamine synthetase

The K_m values of the three forms of the enzyme, for glutamate, ATP and hydroxylamine were determined. The concentrations of the antibody were chosen such that the "protected" and the inhibited enzymes had identical V_{max} values.

Table 2. Protection by substrates of mung bean glutamine synthetase activity against inhibition by its antibody.

Preincubation with	Specific activity (μmol/min/mg protein)	Per cent inhibition
None*	13	0
Antibody**	3·8	71
Glu + ATP + Mg^{2+} + Antibody[a]	7·2	45
ATP + Mg^{2+} + Antibody[b]	4·6	64
Glu + Antibody[c]	4·0	70
NH_2OH + Antibody[d]	3·8	71
Glu + NH_2OH + Antibody[e]	3·7	72
NH_2OH + ATP + Mg^{2+} + Antibody[f]	4·0	70

* The enzyme activity in the absence of antibody.

** The enzyme preincubated with the antibody (150 μg) for 5 min at 37° C prior to a second preincubation with the substrates.

The enzyme (5–6 μg) was preincubated separately with the saturating concentrations of *a*. glutamate and ATP, *b*. ATP (10 mM), *c*. glutamate (100 mM), *d*. hydroxylamine (10 mM), *e*. glutamate and hydroxylamine, *f*. hydroxylamine and ATP, for 5 min at 37° C. This was followed by a second preincubation with the antibody (150 μg) under identical conditions. The reaction was started by the addition of substrate(s) which was not present in the preincubation medium. The enzyme in the absence of antibody pre-incubated as above served as the control and the activity was normalised to 100..

Table 3. Minimum concentration of glutamate required for protection of glutamine synthetase against inhibition by antibody.

Preincubation with	Per cent inhibition
None*	0
Antibody	50
ATP-Mg (10 mM) + Antibody	50
Glu (0·1 mM) + ATP–Mg (10 mM)$^{2+}$ + Antibody	56
Glu (0·5 mM) + ATP–Mg (10 mM)$^{2+}$ + Antibody	56
Glu (1 mM) + ATP–Mg (10 mM)$^{2+}$ + Antibody	36
Glu (2 mM) + ATP–Mg (10 mM)$^{2+}$ + Antibody	26
Glu (5 mM) + ATP–Mg (10 mM)$^{2+}$ + Antibody	26
Glu (100 mM) + ATP–Mg (10 mM)$^{2+}$ + Antibody	26

* Enzyme activity in the absence of antibody, normalised to 100 The enzyme was preincubated with a mixture of ATP (10 mM, saturating) and glutamate (0·1 to 100mM) for 5 min at 37° C followed by a second preincubation with the antibody (50 μg). The enzyme activity was measured at saturating concentrations of glutamate and hydroxylamine,

Table 4. Minimum concentration of ATP required for protection of glutamine synthetase activity against inhibition by antibody.

Preincubation with	Per cent inhibition
None*	0
Antibody	50
Glutamate (100 mM) + Antibody	50
ATP–Mg^{2+} (0·05 mM) + Glu (100 mM) + Antibody	22
ATP–Mg^{2+} (0·1 mM) + Glu (100 mM) + Antibody	20
ATP–Mg^{2+} (0·2 mM) + Glu (100 mM) + Antibody	27
ATP–Mg^{2+} (5 mM) + Glu (100 mM) + Antibody	27
ATP–Mg^{2+} (10 mM) + Glu (100 mM) + Antibody	27

* Enzyme activity in the absence of antibody normalised to 100. The enzyme was preincubated with a mixture of glutamate (100 mM) and ATP (0·05 mM–10 mM) for 5 min at 37° C prior to preincubation with the antibody (50 *μg).* The enzyme activity was measured at saturating concentrations of ATP and hydroxylamine.

Figures 5, 6 and 7 depict the Lineweaver-Burk plots for glutamate, ATP and hydroxylamine, respectively. The K_m values of the inhibited enzyme for glutamate (6·25 mM) was 25 fold higher than that for the native enzyme while the K_m values for ATP and hydroxylamine were two-fold greater than those for the native

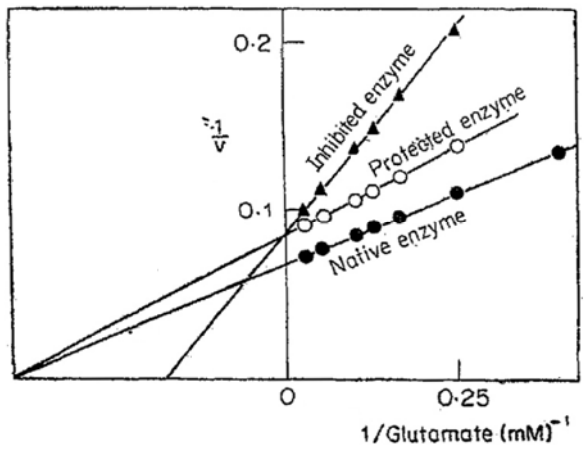


Figure 5. Double reciprocal plot of velocity versus glutamate concentration. (–●–●–) Native enzyme; (5-6*μ*g); (– O – O –) ; Protected enzyme.
The enzyme preincubated with 2 mM glutamate and 10 mM ATP prior to the addition of antibody (30 *μ*g). Glutamate concentration was varied in the reaction mixture from 2-50 mM and the reaction was started by the addition of saturating concentration of hydroxylamine. (–▲–▲–) The enzyme was preincubated with the antibody for 5 min at 37° C followed by a second preincubation with glutamate concentrations indicated in figures and ATP (10 mM). The reaction was started by the addition of saturating concentration of hydroxylamine,

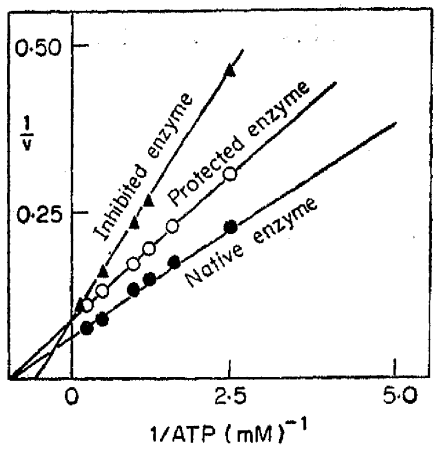


Figure 6. Double reciprocal plot of velocity versus ATP concentration (–● – ● –) Native enzyme; (– O – O –) Protected enzyme.
The enzyme was preincubated with 100 mM glutamate and 0·1 mM ATP prior to the addition of antibody (30 μg). ATP concentration was varied in the reaction mixture from 0·5–10 mM and the reaction was started by the addition of saturating concentration of hydroxylamine (10 mM). (–▲–▲–). The enzyme was preincubated with the antibody for 5 min at 37° C followed by a second preincubation with ATP (concentrations indicated in figure 6) and glutamate (100mM) The reaction was started by the addition of saturating concentration of hydroxylamine.

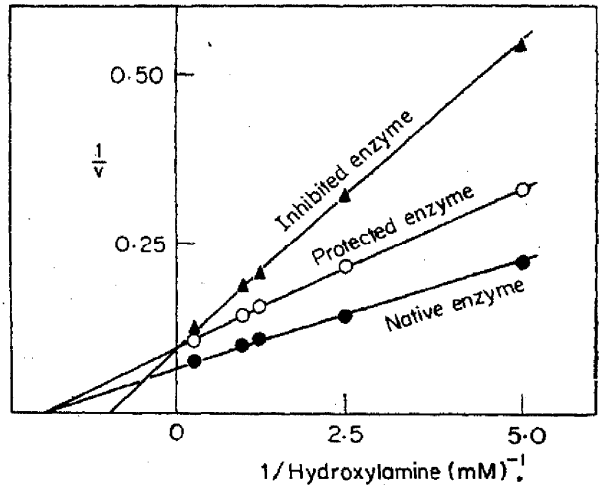


Figure 7. Determination of K_m value for hydroxylamine.
(–● –●–) Native enzyme ; (– O – O –) protected enzyme.
[The enzyme preincubated with saturating concentrations of glutamate and ATP prior to the addition of antibody]. The reaction was started by the addition of varying amounts of hydroxylamine indicated in the figure. (–▲–▲–) inhibited enzyme. [The enzyme preincubated with the antibody prior to the addition of the substrates]. The reaction was started by the addition of varying amounts of hydroxylamine indicated in the figure.

enzyme (2 and 1 mM). On the contrary, the protected enzyme had the same K_m values for the substrates as those for the native enzyme.

Discussion

Glutamine synthetase from *Phaseolus aureus* seedlings was purified to homogeneity. The molecular weight reported for mung bean glutamine synthetase (775,000) was larger than the value reported for glutamine synthetase from other sources. The mung bean enzyme consisted of identical subunits of molecular weight 50,000 which was comparable to that reported from other sources. Thus, the plant as well as the mammalian and bacterial enzymes are composed of subunits whose molecular weights fall within the narrow range of 44,000 to 50,000 (Tate and Meister, 1973).

The mung bean glutamine synthetase activity was completely inhibited by its antibody. The enzyme activity could be partly protected against inhibition by preincubation of the enzyme with glutamate and ATP. The concentration of antibody required for 50% inhibition of enzyme activity was 0·17 μM (the molecular weight of rabbit IgG was assumedto be 150,000, Putnam, 1962) for the native enzyme and 0·78 μM for the protected enzyme. In the presence of infinite concentrations of antibody, while the native enzyme was completely inactive, the protected enzyme retained 20% of the enzyme activity (inset figure 4) The residual enzyme activity might be due to the stabilisation of the active site of the protected enzyme or decreased affinity of the enzyme for the antibody as a result of the altered conformation of the antigenic determinant due to the binding of ATP and glutamate to the enzyme. Protection, by substrates, of the enzyme activity against inhibition by antibody was also reported for muscle glycogen phosphorylase (Michaelides *et al.,* 1964).

The change in K_m values of the inhibited enzyme for the substrates may be explained as follows. The heterogeneous population of antibodies contained in the γ-globulin fraction of mung bean glutamine synthetase antiserum could be envisaged to have differential affinities for their binding sites on the enzyme, and hence, until all the antigenic determinants were masked, the enzyme activity persists. The binding of the antibody at an antigenic determinant close to the active site could prevent the substrate interaction, causing inhibition. Alternatively, binding at the antigenic determinant site could cause a conformational change in the protein leading to inaccessibility of the active site to the substrate When some of the binding sites were not occupied, the resulting enzyme-antibody complexes might be active with altered affinities for the substrates. When subsaturating concentrations of the antibody were added to the enzyme, prior to the addition of the substrates, the mixture would consist of completely inactive enzyme-antibody complex, partially active enzyme-antibody complex, and the unreacted native enzyme. The partially active enzyme-antibody complex might be responsible for the altered K_m values due to its decreased affinities for the substrates It would have been preferable if the inactive enzyme-antibody complex was separated from the unreacted enzyme and partially active enzyme-antibody complex and characterised physically.

The absence of any change in the K_m value of the protected enzyme for the substrates might be due to a substrate-induced conformational change in the enzyme

affecting the enzyme-antibody reactions. The resulting enzyme-antibody complex could be partially active with the K_m value similar to the native enzyme. Alternatively, among the antibody species, one of them could interact with the altered enzyme conformation to yield a completely active enzyme-antibody complex while the rest of the reactions led to inactive complexes, As this complex formed only a small portion of the enzyme-antibody complexes, a decrease in V_{max} was observed without a change in K_m. Further experiments with the purified antibody are necessary to establish which of these possibilities are correct.

These results indicate that the binding of ATP and glutamate to the enzyme induced a conformational change and that the antigen-antibody reactions can be meaningfully employed to probe these changes. This approach can be extended to the study of the interactions of the regulators with the mung bean glutamine synthetase.

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