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Studies on *Aspergillus niger* glutamine synthetase: Regulation of enzyme levels by nitrogen sources and identification of active site residues

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Abstract. The specific activity of glutamine synthetase (L-glutamate: ammonia ligase, EC 6.3.1.2) in surface grown *Aspergillus niger* was increased 3-5 fold when grown on L-glutamate or potassium nitrate, compared to the activity obtained on ammonium chloride. The levels of glutamine synthetase was regulated by the availability of nitrogen source like NH_4^+, and further, the enzyme is repressed by increasing concentrations of NH_4^+. In contrast to other micro-organisms, the *Aspergillus niger* enzyme was neither specifically inactivated by NH_4^+ or L-glutamine nor regulated by covalent modification.

Glutamine synthetase from *Aspergillus niger* was purified to homogenity. The native enzyme is octameric with a molecular weight of 385,000±25,000. The enzyme also catalyses Mn^{2+} or Mg^{2+}-dependent synthetase and Mn^{2+}-dependent transferase activity.

Aspergillus *niger* glutamine synthetase was completely inactivated by two mol of phenylglyoxal and one mol of N-ethylmaleimide with second order rate constants of 3·8 M^{-1} min^{-1} and 760 M^{-1} min^{-1} respectively. Ligands like Mg. ATP, Mg. ADP, Mg. AMP, L-glutamate NH_4^+, Mn^{2+} protected the enzyme against inactivation. The pattern of inactivation and protection afforded by different ligands against N-ethylamaleimide and phenylglyoxal was remarkably similar. These results suggest that metal ATP complex acts as a substrate and interacts with an arginine ressidue at the active site. Further, the metal ion and the free nucleotide probably interact at other sites on the enzyme affecting the catalytic activity.

Keywords. *Aspergillus niger;* glutamine synthetase; nitrogen regulation; purification; kinetic properties; active site residues.

Introduction

Glutamine functions as a nitrogen donor for a number of nitrogenous end products. Glutamine synthetase catalyzing the formation of L-glutamine has been established to be a key regulatory enzyme in several Gram negative bacteria (Stadtman and Ginsburg, 1974; Tyler, 1978). Although a few studies on this enzyme have been carried out with eukaryotic micro-organisms (Limon-Lason *et al.,* 1977; Quinto *et al.,* 1977., Mora *et al.,* 1980; Ferguson and Sims, 1974a; Generalova and Abramova, 1975; Legrain *et al.,* 1982), not much is known on the mechanisms of regulation of this enzyme activity.

Abbreviations used: DEAE, Diethylaminoethyl; Buffer A, 20 mM imidazole-HCl buffer, pH 7·5 containing 0·1mM EDTA, 2mM 2-mercaptoethanol and 5% glycerol; Glu, L-glutamate; γGHA, γ-glutamylhydroxamate; NEM, N-ethylmaleiimide; *p*HMB, *p*-hydroxymercuribenzoate; Gln, L-glutamine; EDC, 3-(3-dimethylaminopropyl)-l-ethyl-carbodiimide; SDS, sodium dodecyl sulphate; Tris, Tris(hydroxymethyl)aminomethane, M_r, molecular weight; k_{app}, pseudo first order rate constant.

Aspergillus niger is extensively used in the manufacture of citric acid by fermentation. This derangement of carbon metabolism has been attributed to an alteration in the activities and synthesis of the enzymes of tricarboxylic acid cycle (Kubicek and Rohr, 1978; Bowes and Mattey, 1980). However these results do not completely explain the physiology of citric acid fermentation. For the following reasons, we hypothesize that a marked decrease in the activity of glutamine synthetase under conditions favourable for citric acid excretion, would result in shutting down of the nitrogen metabolism and consequently backing up of the carbon metabolism at the tricarboxylic acid cycle level: (a) Glutamine synthetase via glutamate dehydrogenase links tricarboxylic acid cycle and nitrogen metabolism; (b) Conditions used for maximal citric acid production are, poor buffering and high acidity of the medium, situations which are unfavourable for optimal activity of enzymes; (c) Manganese deficiency is an essential prerequisite for optimal citric acid production and glutamine synthetase is one of a few enzymes requiring manganese. As an initial step in testing this hypothesis, a study of the regulation of the enzyme levels and its activity in *A. niger* grown in different nitrogen sources was undertaken.

Materials and methods

All the chemicals used were of analytical grade or purchased from Sigma Chemical Company, St. Louis, Missouri, USA, except DEAE-Sephacel and Sepharose-4B which were from Pharmacia Fine Chemicals, Uppsala, Sweden; Biogel A5M was from Biorad Laboratories, Richmond, California, USA.

Organism and growth conditions

A. niger (UBC 814) was grown as surface cultures in one liter flasks containing 100 ml of the culture medium. The medium composition was essentially the same as described for *A. nidulans* by Pateman (1969) with minor modifications. Addition of micronutrients (mg/litre); $FeCl_3$ $6H_2O$, 20; $ZnSO_4$ $7H_2O$, 10; $MnSO_4$ H_2O, 3; Na_2MoO_4 $2H_2O$, 1·5; $CuSO_4 \cdot 5H_2O$, 1·0; improved the growth and yield of cells. One per cent glucose was used as carbon source. Nitrogenous compounds were used at 100 mM concentration, unless otherwise mentioned.

Uniform spore suspension (3 ml) obtained from 7–10 day old cultures was used as inoculum. The cells were harvested in the maximal growth phase (45th after inoculation), washed and stored at – 20°C.

Preparation of cell extracts

Frozen fungal mat was crushed with equal amounts (wt/wt) of fine glass powder and extracted with 5 vol of 20 mM 2-mercaptoethanol and 5% glycerol (Buffer A). The clear supernatant obtained after centrifugation at 12,000*g* for 20 min in a Sorvall RC-5B refrigerated centrifuge, was designated as the crude extract. All the operations were carried out at 0–4°C.

Assay of glutamine synthetase

The enzyme was assayed by the colourimetric determination of *y*-glutamylhydroxamate (*y*GHA) formed (Lipmann and Tuttle, 1945). The reaction mixture (0·5 ml) for estimating Mg^{2+}-dependent synthetase activity contained; 100 mM monosodium L-glutamate (Glu), 50 mM hydroxylamine-HCl (freshly neutralized with 1 M NaOH), 10 mM ATP, 20 mM $MgCl_2$ and 100 mM imidazole-HCl buffer, pH 7·8. The components for the Mn^{2+}-dependent synthetase activity of the enzyme were same as those for the Mg^{2+}-dependent activity except that 4 mM $MnSO_4$ replaced 20 mM $MgCl_2$ and was assayed at the optimum pH of 5·5.

The reaction mixtures (0·5 ml) for the assay of Mn^{2+}-dependent *y*-glutamyl transferase activity contained; 112·5 mM L-glutamine (Gln), 50 mM hydroxylamine-HCl, 20 mM sodium arsenate, 1 mM $MnSO_4$, 0·5 mM'ADP and 100 mM imidazole-HCl buffer pH 6·0.

The reaction was started by the addition of an appropriate amount of enzyme and incubated for 15 min at 28°C. The amount of *y*GHA formed was estimated (Rowe *et al.,* 1970). One unit of enzyme was defined as the amount that catalyzed the formation of one μmol of *y*GHA per min. Specific activity was expressed as units/mg protein. Protein was estimated according to Lowry *et al.* (1951) using crystalline bovine serum albumin as the standard.

Preparation of AMP-Sepharose

Aminohexane-Sepharose prepared as described by March *et al.* (1974), was washed thoroughly with glass distilled water. Disodium salt of AMP (1 mmol) was pre-incubated with 2·5 mmol of 3-(3-dimethylaminopropyl)-1-ethyl-carbodiimide (EDC) for 10 min at 60°C in 3 ml of distilled water and then added to a stirred suspension of (10 ml packed volume) the gel and 7 ml of distilled water. The reaction mixture was stirred gently for 18 h at room temperature and for a further 24 h at 0-4°C. At the end of the reaction, the gel was washed with water and stored at 4°C. The incorporation of the ligand attached by a phosphonamide linkage was measured by the method of Failla and Santi (1973).

Chemical modification of amino acid residues at the catalytic site of A. niger glutamine synthetase

For N-ethylmaleiimide (NEM) inactivation, the enzyme was made essentially free of 2-mercaptoethanol by passing it through a G-25 column (1 × 5 cm) just before use. The inactivation of the enzyme in 50 mM imidazole-HCl buffer, pH 7·5 by either phenylglyoxal or NEM was carried out at 28°C by incubating the enzyme (40–60 μg) with appropriate concentrations of the reagent in a scaled up reaction mixture (500 μl) and withdrawing aliquots (50 μl) at regular time intervals indicated. The reaction was terminated by diluting the enzyme into assay mixture directly. It was ensured that the chemical modification reaction was not occurring during the time required for estimating the enzyme activity. The velocity of the enzyme catalyzed reaction at zero

time *i.e.*, immediately after the addition of inactivating reagent, was normalized to 100 and the residual activity was expressed as per cent of this normalized value. During inactivation of glutamine synthetase by either phenylglyoxal or NEM, there was a parallel loss of both Mg^{2+}-dependent synthetase and Mn^{2+}-dependent transferase activity and the protective influence of various ligands on the two activities was identical for both the inactivations. Hence, the enzyme activity was conveniently monitored by *y*-glutamyl transferase assay. The effect of various ligands on modification was checked by including them in the inactivating system at appropriate concentrations.

Results

Correlation between growth on different nitrogen sources and glutamine synthetase levels

Figure 1 shows the growth curve for Glu-grown (100 mM) *A. niger*. Both specific and total activities of glutamine synthetase were highest around the maximal growth phase (45 h). A rapid decline in the levels of this enzyme occurred just before the onset of conidiation. The ratio of Mg^{2+}-dependent glutamine synthetase to *y*-glutamyl transferase activity remained constant (0·12–0·18) during different stages of growth. Similar pattern of growth and changes in enzyme activity were obtained with L-glutamine (Gln), potassium nitrate and NH_4^+. However, with nitrate the yield of cells was decreased by about 50%.

When Glu (100 mM) or NO_3^- (100 mM) was used as the nitrogen source, the specific

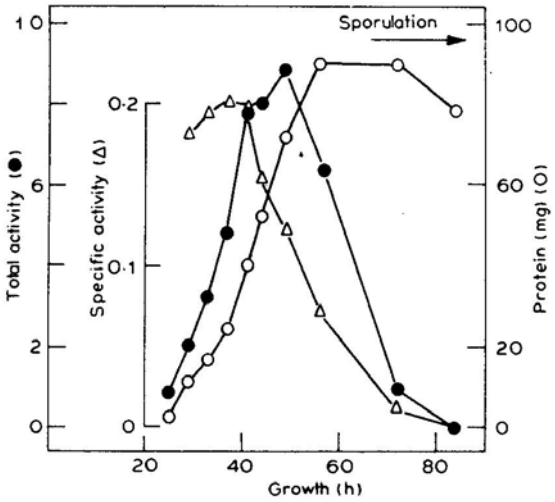

Figure 1. Growth curve and the levels of glutamine synthetase in *A. niger* grown on 100 mM Glu. The cells were harvested at time intervals indicated and the protein content, (O); total (●) and specific (Δ)Mg^{2+} -dependent glutamine synthetase activity in the crude extracts prepared as described in materials and methods is presented. Similar results were obtained when the Mn^{2+}-dependent *y*-glutamyl transferase activity was monitored.

activity of the enzyme at 45 h was 0·096 and 0·135 respectively, compared to the values in the range 0·023-0-036 obtained when Gln (50 mM), L-asparagine (50 mM), urea (50 mM) and NH_4^+ (100 mM), were used as the nitrogen sources. In all the cases, increasing the concentration of nitrogen source from 50 mM to 100 mM resulted in a decrease in the specific activity of the enzyme. The ratio of Mg^{2+} -dependent glutamine synthetase to *y*-glutamyl transferase activity remained essentially constant (0·14-0·12) irrespective of the nature of the nitrogen source. In order to determine whether Glu was specifically inducing the enzyme or not, the levels of this enzyme were monitored in cells grown on media containing a mixture of nitrogenous compounds (table 1). Even under conditions for the enzyme induction, *e.g.,* Glu (100 mM), addition of a small amount of a second, preferred nitrogen source for *e.g.,* Gln (10 mM) or NH^+_4 (10 mM) decreased the activity of the enzyme to near basal levels. Similar results were obtained with NO_3^- grown cells (data not presented), but the concentration of Gln or NH^+_4 (30 mM) required were slightly higher. A direct experiment to check the effect of varying concentrations of NH^+_4 on the levels of this enzyme was carried out (table 2). The concentration of the nitrogen source had an inverse relationship with the levels of

Table 1. The effect of a second nitrogen source on the glutamine synthetase levels in *A. niger* grown on Glu.

Nitrogen Source (100 mM Glu)	Specific[a] activity	Ratio[b]
Control	0·097	0·16
+ NH_4Cl (10 mM)	0·046	0·16
+ KNO_3 (10 mM)	0·083	0·16
+ L-Glutamine (10 mM)	0·045	0·14
NH_4Cl alone (100 mM)	0·027	0·16
Methylamine alone (100 mM)	0·051	0·16

[a] Mg $^{2+}$-dependent glutamine synthetase activity, units/mg protein.
[b] Ratio of Mg^{2+}-dependent glutamine synthetase to Mn^{2+}-dependent *y*-glutamyl transferase activity.

Table 2. Regulation of glutamine synthetase levels by NH_4^+ concentration in the medium.

NH_4Cl (mM)	Specific activity*
10	0·059
30	0·051
50	0·037
70	0·029
90	0·026
100	0·027

* Mg^{2+}-dependent glutamine synthetase activity.

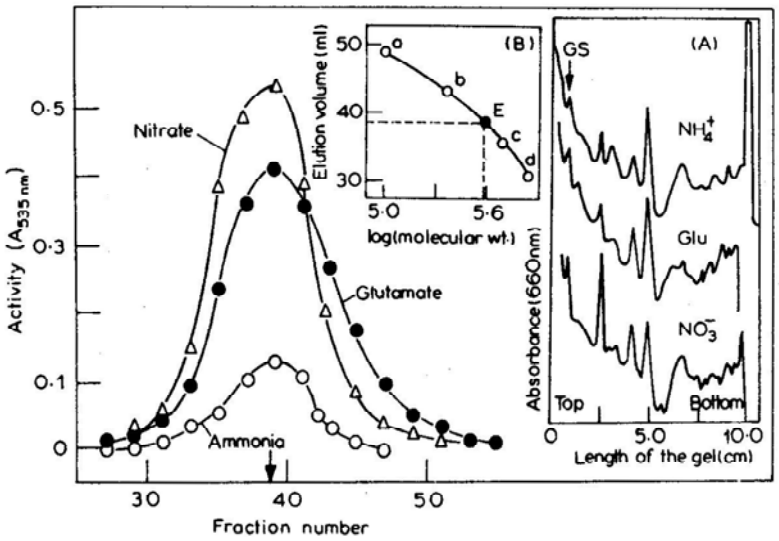

Figure 2. Biogel A5M gel filtration profiles of glutamine synthetase in crude extracts of *A. niger* grown on NH_4^+, Glu and NO_3^-. The arrow indicates peak position.

A. Analytical Polyacrylamide gel electrophoresis of crude glutamine synthetase. The crude extracts (200μg protein) obtained from cells grown on NH_4^-, Glu or NO_3^- were subjected to analytical Polyacrylamide gel electrophoresis at 4°C, in 7·5 % Polyacrylamide gels in 0·5 M Tris-0·39 M glycine buffer, pH 8·6, at a constant current of 2 mA per tube for 3 h (Davis, 1964). One set of gels were stained for protein using Coomassie brilliant blue G (0·02 % in 3·5 % perchloric acid) and a second set of gels were sliced into 0·5 mm bits and the protein was extracted into the assay buffer by maceration and enzyme activity was measured. The stained gels were scanned at 660 nm in a Beckmann Model-26 Spectrophotometer. The arrow indicates the position of glutamine synthetase activity.

B. The M_r of the native enzyme was determined by the method of Andrews (1965), using Biogel A5M column (0·6×65 cm) equilibrated with buffer A and operated at a flow rate of 10–12 ml/h. The marker proteins (2-5 mg each, in a total volume of 0·5 ml) were loaded separately and 0·75 ml fractions were collected. The elution of hexokinase (a, 102,000), catalase (b, 232,000), ferritin (c, 480,000) and thyroglobulin (d, 669,000) were monitored by measuring the absorbance at 280 nm. The numbers in parenthesis indicate M_r of markers. Glutamine synthetase (E) was chromatographed separately and its elution profile was monitored by assaying the Mn^{2+}-dependent transferase activity. The M_r of the enzyme was calculated by extrapolation to be 385,000±25,000. Both the purified enzyme (150 μg) and the enzyme present in the crude extracts were extruded from the column at identical volumes.

glutamine synthetase. Higher the concentration of nitrogen source, lower was the glutamine synthetase specific activity. Similar results were obtained with urea, Gln, Glu, L-asparagine, and NO_3^-.

It was observed that the molecular and catalytic properties of glutamine synthetases were dependent on the nitrogen source in the growth medium (Vichido *et al.,* 1978; Sims *et al.,*1974b). In order to examine whether any such changes were occurring in the case of *A. niger* enzyme, the organism was grown on Glu, NH_4^+ or NO_3^- (all at 100 mM) and the activity of the enzyme in the crude extracts as well as its electrophoretic mobility (figure 2A) and elution profile on gel filtration (figure 2) was determined. The intensities

of the dye stain corresponding to glutamine synthetase protein were roughly proportional to the activity measured in the crude extracts. However, the mobility of glutamine synthetase in all the three cases was similar. Also, the enzyme from these cases eluted from the gel filtration column (Biogel A5M, 0·6×65 cm) as a single symmetrical peak with same elution volume corresponding to a molecular weight (*Mr*) of 385,000±25,000 (figure 2B).

Absence of regulation by rapid inactivation

In order to determine whether the modulation of enzyme activity *in vivo* was occurring by enhanced degradation of the enzyme or by its covalent modification as in *Escherichia coli* (Stadtman and Ginsburg, 1974), cells harvested at maximal growth phase (45 h) on Glu were transferred on to Gln (100 mM) or NH^+_4 (100 mM) medium and glutamine synthetase activity was measured. The activity decreased by only about 50-60% over 4–5 h (figure 3). This suggested that there was no rapid and specific in *vivo* glutamine synthetase inactivating system. Final glutamine synthetase levels to which the organism adjusted were comparable to the basal levels of the enzyme obtained when grown on Gln or NH^+_4. The ratio of Mg^{2+} -dependent glutamine synthetase to *y*-glutamyl transferase activity remained constant (0·14–0·16) at each time point.

When the crude extract from *A. niger* cells grown on Gln and NH^+_4 were mixed with the extracts of glutamate-grown cells (45 h), incubated upto 30 min at room temperature (28°C) and monitored for glutamine synthetase activity, there was no loss of activity in the combined extracts (data not presented).

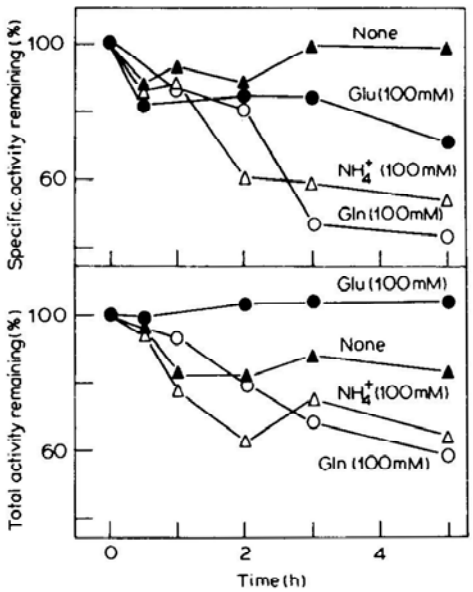

Figure 3. Inactivation of glutamine synthetase *A. niger* was grown on 100 mM Glu for 45 h, harvested, washed aseptically and transferred on to a medium containing no nitrogen source, (▲);Glu, (●); Gin, (O); and NH_4^+ (Δ) (all at 100 mM). The total and specific activities were determined at the time intervals indicated after transfer. The 100 *%* values correspond to a *y*-glutamyl transferase specific activity of 0·647.

Purification of glutamine synthetase

Cells grown on 66 mM Glu as the sole source of nitrogen were used as the starting material. The enzyme was stabilized against inactivation during purification by including 2-mercaptoethanol, EDTA and 5 % glycerol in the buffer (Buffer A) during all the steps of purification. The purification was carried out at 0–4°C. Frozen fungal mat (150 g) crushed with an equal amount (wt/wt) of fine glass powder, extracted with 3 vol of buffer A, was passed through two layers of cheese cloth and the filtrate centrifuged at 12,000 *g* for 20 min in a Sorvall RC-5B refrigerated centrifuge. The supernatant (crude extract, table 3) was treated with protamine sulphate (400 mg), and the precipitated nucleoproteins were removed by centrifugation. To this supernatant, solid ammonium sulphate was added to 45% saturation and the precipitate formed was discarded. The precipitate obtained on raising the saturation to 60 % was collected, dissolved in a small amount of buffer A and desalted by gel filtration on a Sephadex G-25 column (2×30 cm). This fraction (ammonium sulphate fraction, table 3) was loaded on to a DEAE-Sephacel column (2×15 cm) equilibrated with buffer A. The column was washed with the same buffer until the eluate absorbance (at 280 nm) was less than 0·05. A linear KCl gradient (0–0·5 M, 200 ml) at a flow rate of 20 ml/h was used to elute the enzyme from the column. Fractions (2 ml) having maximal activity were pooled (DEAE-Sephacel fraction, table 3). The pooled enzyme was diluted with buffer A to a KCl concentration less than 50 mM and was applied on to the AMP-Sepharose column (1·5 × 6 cm) at a flow rate of 12 ml/h. The unadsorbed protein fraction had no enzyme activity. The column was washed with buffer A till all the unadsorbed protein was leached out of the column. The enzyme was eluted from the column with the same buffer containing 5 mM ATP (neutralized to pH 7·0 with 2 M NaOH) . Fractions (1 ml) were collected, assayed for enzyme activity and active fractions (specific activity>15·0) were pooled (AMP-Sepharose enzyme, table 3). The enzyme obtained from the previous step was applied on to a Sepharose-4B column (1·2×90cm) which was previously equilibrated with buffer A. Fractions (1 ml) were collected and assayed for the enzyme activity. Those fractions having more than 50% activity of the peak fraction were pooled (Sepharose-4B fraction, table 3).

Table 3. Purification of glutamine synthetase from *A. niger*.

Step	Total activity (Units)[a]	Specific activity (Units/mg)	Fold purification	Per cent recovery
Crude	841	0·4	1	100
$(NH_4)_2SO_4$ (45–60%)	631	1·4	3	75
DEAE-Sephacel	457	4·1	10	54
AMP-Sepharose	185	18·7	46	22
Sepharose 4B	67	19·8	49	8

Weight of the fungal mat used = 150 g.

[a] μmol of *y*GHA formed/min at 28°C. The Mn^{2+}-dependent *y*-glutamyl transferase activity was monitored.

A summary of the purification procedure is given in table 3. This procedure resulted in a 50-fold purification of the enzyme with 8 % recovery. The recoveries and specific activity of the enzyme varied somewhat from preparation to preparation and depended on the fractions that were pooled in the last two steps of purification. The final gel filtration step was essential to remove trace contaminants. The enzyme thus obtained was divided into small volumes and stored at –40°C. Freshly thawed enzyme was used in each experiment.

Physico-chemical and kinetic properties of the enzyme

The final preparation (table 3) showed a single band (figure 4C) on the disc gel electrophoresis (Davis, 1964) in Tris(hydroxymethyl)aminomethane (Tris)-glycine buffer pH 8·6, and the enzyme activity peak coincided well with the protein band observed on staining the gel with Coomassie brilliant blue G (figure 4). From standard curve (figure 2, Inset B) drawn using thyroglobulin (669,000), ferritin (480,000), catalase (232,000) and hexokinase (102,000) as marker proteins, the M_r of the native, *A. niger* glutamine synthetase was calculated to be 385,000±25,000. The subunit M_r of the enzyme was obtained from sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis using bovine serum albumin (67,000), ovalbumin (43,000) and α-chymotrypsinogen (24,000) as markers (Weber and Osborn, 1969). The enzyme gave a single protein band corresponding to a M_r of 53,000 ± 5,000 (figure 4, Inset D).

The Mn^{2+}- and Mg^{2+}-dependent glutamine synthetase activities were linear with enzyme concentration upto 18μg and 10μg, respectively. The Mn^{2+} -dependent *y*-glutamyl transferase activity of the enzyme was linear upto 7 μg protein. All the three activities were linear with time upto 20 min and the enzyme activities were routinely assayed for 15 min. The Mn^{2+}-supported glutamine synthetase activity functioned optimally at 33°C. The Mg^{2+}-dependent synthetase activity had a pH optimum around 7·8, whereas the Mn^{2+}-supported synthetase activity was maximal at pH 5·5 and the

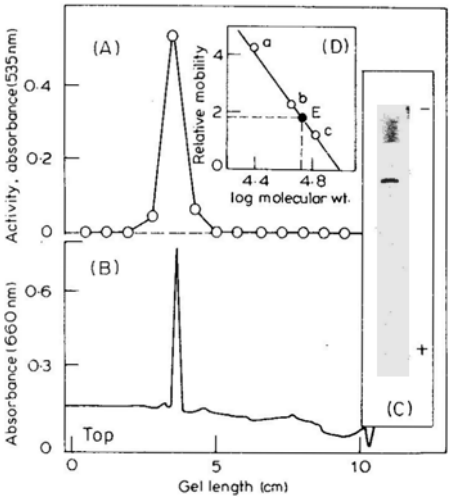

Figure 4. Polyacrylamide gel electrophoresis of the purified *A. niger* glutamine synthetase (100 μg of the enzyme used. Details of the procedure are given in legend to figure 2). After electrophoresis the gel was sliced into 0·5 mm pieces, the enzyme extracted by maceration in buffer A (0·2 ml). The reaction was started by the addition of substrates and *y*-glutamyl transferase activity was measured (A). A duplicate gel was stained and the absorbance easured at 660 nm (B). The gel photograph is shown as inset C. The M_r was determined by extrapolation from the standard curve (inset D).

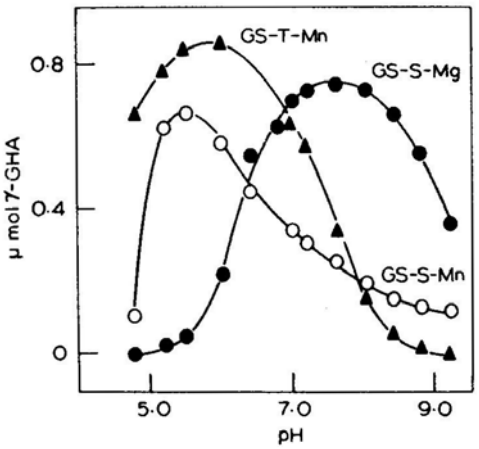

Figure 5. pH Optima of *A. niger* glutamine synthetase activities.

The Mg^{2+}-dependent synthetase, (●); the Mn^{2+}-dependent synthetase, (O); and the *y*-glutamyl transferase, (▲), activities were determined using acetic acid/sodium acetate (pH 4·8), Tris, maleate-KOH (pH 5·2-6·0) and Tris-imidazole-HCl (pH 6·0-9·2) buffers (all at 100 mM). The buffer ions had no effect on the enzyme activity. The enzyme concentrations used were usually about 8μg protein for synthetase assays and 3 μg for the transferase assay.

Mn^{2+}-dependent *y*-glutamyl transferase reaction of the enzyme showed a pH optimum of 6·0 (figure 5).

Glutamine synthetase from *A. niger* could use either N H^{+}_{4} or NH_2OH as the substrate. The enzyme was about 50 % active when Glu was replaced by D-glutamate in the assay. Of the various metal ions tested (Co^{2+}, Cr^{2+}, Ca^{2+}, Fe^{2+}, Sr^{2+}, Ba^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+} and Mn^{2+}) only Mn^{2+} and Mg^{2+} were effective as activators of the enzyme. The nucleotide triphosphate requirement of the enzyme was quite specific as CTP, GTP, ITP, TTP and UTP could not replace ATP, in the biosynthetic reaction.

Typical hyperbolic saturation curves (figures not given) were obtained with Glu, NH_2OH and ATP (at 20 mM $MgCl_2$). At a fixed concentration of Glu (100 mM) the effect of varying concentrations of NH_2OH (0–50 mM) on the enzyme activity was determined. The maximal velocity was observed at 10 mM NH_2OH. The effect of varying concentrations of Glu (0–150 mM) on the enzyme activity was studied at a fixed concentration of NH_2OH (50 mM). The activity was maximal around 100 mM Glu concentration. The concentration of ATP required for maximum enzyme activity was determined by studying the effect of varying concentrations of ATP on the reaction velocity at a constant and saturating concentration of Glu and NH_2OH. The velocity was highest at a concentration of 10 mM ATP when a 2-fold excess of Mg^{2+} was used. The K_m values for the substrates of Mg^{2+}-dependent synthetase activity *viz.Glu,* NH_2OH and ATP were calculated from the linear Lineweaver-Burk plots after Lee-Wilson (1971) modification and are summarized in table 4. The Mn^{2+}-dependent *y*-glutamyl transferase activity of *A. niger* glutamine synthetase also showed hyperbolic saturation patterns with the substrates Gln and NH_2OH (figures not presented). This activity of the enzyme required optimal concentrations of Mn^{2+} (1 mM), ADP (0·5 mM) and sodium arsenate (20 mM) as nonconsummable substrates. When NH_2OH concentration was varied 0-50 mM at a fixed concentration of Gln (112·5 mM), maximal activity was obtained at 30 mM of NH_2OH. Gln concentration could not be increased beyond 112·5 mM because of the limited solubility of this amino acid in water (saturated solution of Gln in water is about 250 mM). The K_m values for Gln and NH_2OH were obtained by the above mentioned method. The Michaelis constants for all the substrates of the enzyme have been summarized in table 4. The

Table 4. Michaelis constants for different substrates of glutamine synthetase from *A. niger*.

Substrate	K_m (mM)[a]
Mg^{2+}-dependent synthetase activity:	
Glu	10·0
NH_2OH	0·5
ATP (at 20 mM $MgCl_2$)	1·5
Mn^{2+}-dependent γ-glutamyl transferase activity:	
Gln[b]	235
NH_2OH	4·0
Mn^{2+} ($K_{0.5}$ at 0·5 mM ADP)	0·07

[a] The Michaelis constants for various substrates were obtained by applying Lee-Wilson (1971) modification to the double reciprocal plots. This was necessary because, the K_m value for NH_2OH and Mg. ATP were low and hence appreciable amounts of product formation (> 10 %) could not be avoided during the colourimetric assay.

Arithmetic mean of substrate concentration [S] is an excellent approximation of true substrate concentration when substantial fraction of the substrate is utilized during the assay. If [S_o] is the initial substrate concentration and [S] is the substrate remaining at the end of the assay, then the modified Lineweaver-Burk (1934) equation assumes the form,

$$\frac{1}{\bar{v}} = \frac{K_m}{V_m[\bar{S}]} + \frac{1}{V_m}.$$

Where

$$\bar{v} = \frac{[S_o]-[S]}{t} \text{ and } [\bar{S}] = \frac{1}{2}([S_o]+[S]).$$

This procedure provided a more reliable estimate of K_m values. If the reaction goes to 50% completion, the K_m value estimated by this method will be high only by 4 %. The results of the modified Lineweaver-Burk plots were analyzed by least-square curve fitting procedure (linear regression) using a programmable pocket calculator (Texas Instruments SR-51A).

[b] Enzyme could not be saturated due to limited solubility of Gln in water.

reaction velocity at a constant concentration of Gln (112·5 mM), NH_2OH (50 mM) and ADP (0·5 mM) was highest around 0·35 mM Mn^{2+} and from the sigmoid saturation pattern a $K_{0.5}$ value of 70 μM was calculated for Mn^{2+} (table 4).

Figure 6 depicts the effect of pH on the Glu saturation of the Mg^{2+}-supported synthetase activity of the enzyme. It can be seen from the linear Lineweaver-Burk plots that the slopes (K_m / V_m) of the lines increase with decreasing p H. The inset of figure 6 shows a replot of —log K_m versus pH. On drawing a tangent with an unit slope to this curve, a group with a pK_a value of 6·5 was implicated. This pK_a value approximated well with the value reported in the literature for a histidine in proteins.

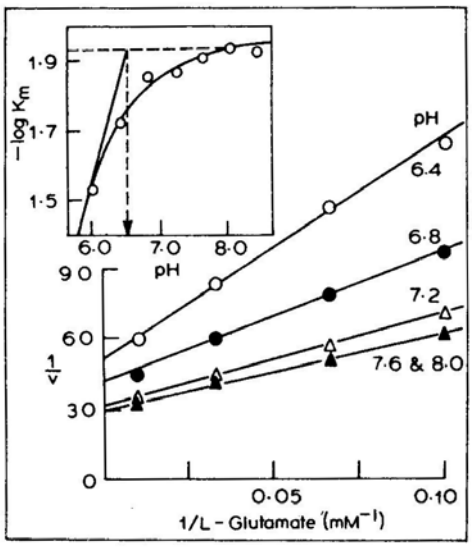

Figure 6. pH Dependence of saturation of the enzyme by Glu.

The Mg^{2+}-dependent synthetase activity was assayed in 100 mM imidazole.HCl buffer, pH 6·0, 6·4, 6·8, 7·2, 7·6, 8.0 or 8·4, at varying concentrations of Glu (0–150 mM) and at a fixed saturating concentration of NH_20H (50 mM) and Mg. ATP (20:10mM). From the Lineweaver-Burk plots, (all the curves are not given to avoid overcrowding but data were used in the calculation of pK_m), the K_m values for Glu were determined at each pH value, by a least-square fit of the data. Inset:- log K_m, obtained at each pH value was plotted against corresponding pH. A pK_m value of 6·5 was calculated.

Functional amino acid residues at the active site

A. niger glutamine synthetase was rapidly inactivated by phenylglyoxal and increasing concentrations of the reagent enhanced the rate of inactivation. Figure 7 depicts the first-order plots obtained from the time course of inactivation at different concentrations of phenylglyoxal. In order to avoid overcrowding of the data, only a few representative curves are shown. A plot of *ln* k_{app} (apparent first-order rate constant) as a function of In [phenylglyoxal] gave a straight line with a slope of 1·6 and from the intercept on the Y-axis, a second-order rate constant of 3·8 M^{-1} min^{-1} was obtained (figure 7, Inset). When glutamine synthetase was incubated with 10 mM phenylglyoxal, it was rapidly inactivated (figure 8A and B). It can be seen from figure 8A that Glu, Mg^{2+} or NH_4^+ had a marginal protective effect on the rate of inactivation whereas both ATP plus $MgCl_2$ and Mn^{2+} afforded considerable protection against phenylglyoxal

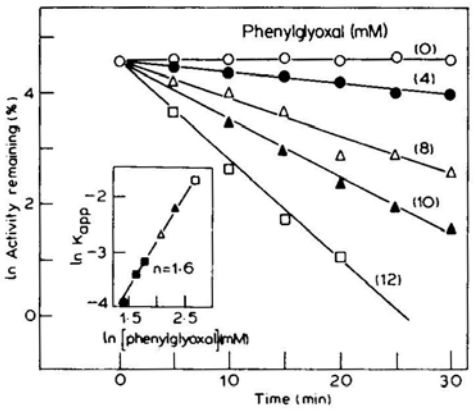

Figure 7. First order plots for the inactivation of the enzyme by phenylglyoxal (O, control; ●, 4mM;■, 5and 6 mM; Δ, 8 mM; 10mM; and □, 12 mM). Inset: The pseudo first order rate constant (k_{app}) at each phenylglyoxal concentration was calculated from the slopes of the first order plots and *ln* k_{app} is plotted against *In* phenylglyoxal concentration. A second order rate constant of 3·8M^{-1} min^{-1} was calculated.

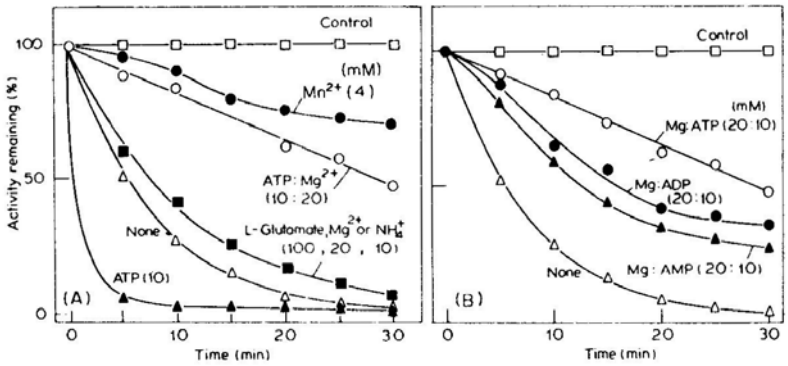

Figure 8. Protection of the enzyme by substrates and adenine nucleotides against phenylglyoxal (10 mM) inactivation.
A. The inactivation fixture in addition to the normal components, (please see materials and methods) contained: no addition (Δ); Mn^{2+} (●, 4 mM); ATP: Mg^{2+} (O, 10:20mM); L-glutamate, Mg^{2+} or NH_4^+ (■, 100, 20, 10 mM, respectively); ATP (▲ 10mM).
B. The inactivation mixture in addition to the normal components contained: no addition (Δ); ATP:Mg^{2+}(O,10:20mM);ADP:Mg^{2+}(●,10:20mM);andAMP:Mg^{2+}(▲,10:20mM). The control curve (□) in both A and B panels shows the absence of inactivation when no phenylglyoxal was added.

inactivation. The presence of free ATP greatly enhanced the inactivation rate. Similarly it is evident from figure 8B that ADP plus $MgCl_2$ and AMP plus $MgCl_2$ also protected the enzyme against inactivation, although the protection was a little less than that afforded by-ATP plus $MgCl_2$. The order of effectiveness of protection by these three ligands was as follows: Mg.ATP > Mg.ADP > Mg.AMP. The Mg. ATP complex protected the enzyme against phenylglyoxal inactivation in a concentration dependent manner as shown by the first-order plots in figure 9A. Similarly, the protection afforded by increasing concentrations of Mn^{2+} (0–100 μM) against phenylglyoxal (10 mM) modification is depicted in figure 9B. From the concentration dependence of protection (figure 9), dissociation constant for the protective ligand was obtained using replots (Nelson *et al.,* 1962). These replots (not shown) gave the dissociation constants of 0·9 mM and 52 μM for Mg.ATP and Mn^{2+}, respectively.

When the enzyme was incubated for 10 min with increasing concentrations of NEM, enhanced loss of activity was observed (figure 10A). A replot of reciprocal of residual activity versus NEM concentration gave a straight line (figure 10A, Inset) indicating that the inactivation followed a first order kinetics and that the activity depended on a single/similar class of site(s) which were susceptible for NEM modification. In a separate experiment, time course of inactivation by increasing concentrations of NEM (0–2 mM) was monitored. From the first order plots (data not shown), k_{app} values were determined and a plot of *In* K_{app} versus *In* [NEM] was made. This plot gave a slope of close to one (0·8) and a second order rate constant of 760 M^{-1} min^{-1} (figure 10B) was calculated. Like in the case of phenylglyoxal, Glu, NH^+_4 or Mg^{2+} did not afford appreciable protection whereas Mn^{2+} and Mg.ATP complex protected the enzyme considerably against the NEM modification (figure not given). The concentration dependence of protection against NEM inactivation by Mn^{2+} is shown in figure 11. By

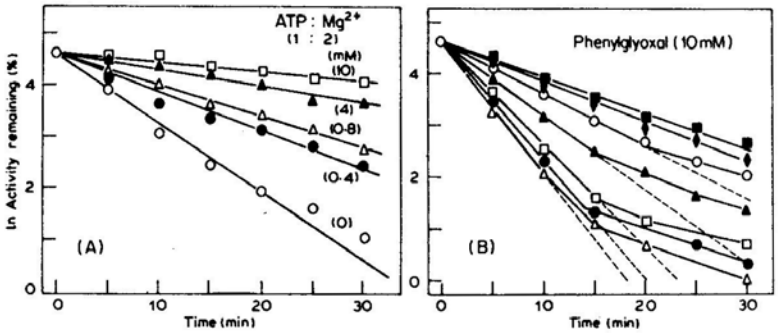

Figure 9. A. First order plots for the protection afforded by ATP: Mg^{2+} (1:2 ratio, O; O mM; ●, 0·4mM; Δ, 0·8mM; ▲, 4mM; and c , 10mM) against inactivation of the enzyme by phenylglyoxal (10 mM).
B. First order plots for the protection afforded by Mn^{2+} (Δ, 0 μM;●, 5 μM; □, 10 µM; ▲, 20 μM; O, 50µM;♦, 70 μM; and ■ 100μM) against inactivation of the enzyme by phenylglyoxal (10 mM).

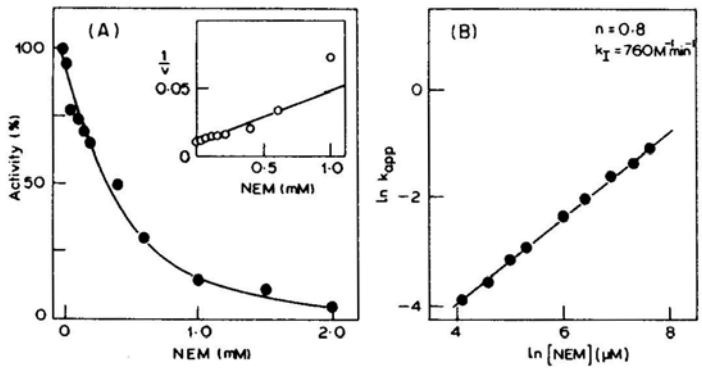

Figure 10. A. Inactivation of the enzyme by increasing concentrations of NEM. Inset: Plot of 1/*v* against NEM concentration.
B. Replot for the determination of second order rate constant and '*n*' value for NEM inactivation.

The time course of the inactivation was carried out at different concentrations of NEM as described for phenylglyoxal (figure 7) and from the first order plots, the k_{app} values were calculated at each concentration of NEM. A second order rate constant of 760 M^{-1} min^{-1} was obtained from the replot. Enzyme without 2-mercaptoethanol was used in all NEM inactivation studies.

suitably replotting this data (as mentioned above) a dissociation constant for Mn^{2+}, of 14 μM was calculated.

The pseudo first order rate constants (k_{app}) for inactivation by both NEM (0·4 mM) as well as phenylglyoxal (10 mM) were determined in the presence of Mn^{2+}, free ATP or different ratios of ATP:Mg^{2+} and are summarized in figure 12. The effect of inclusion of various ligands was remarkably similar on the inactivation rate constants for NEM as well as phenylglyoxal. Whereas Mg. ATP complex and Mn^{2+} protected

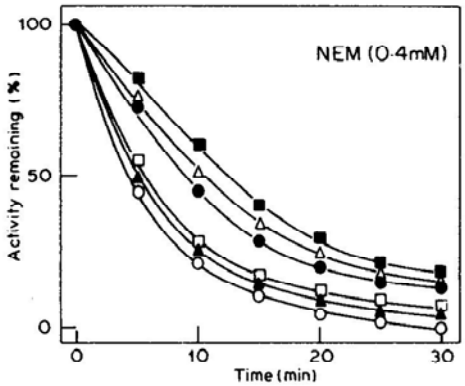

Figure 11. Protection by difference concentrations of Mn^{2+} (O, 0 μM;▲, 3μM; □, 5μ M; ●, 50 μM; Δ, 70 μM and ■, 100 μM) against inactiveation of the enzyme by NEM (0·4 mM).

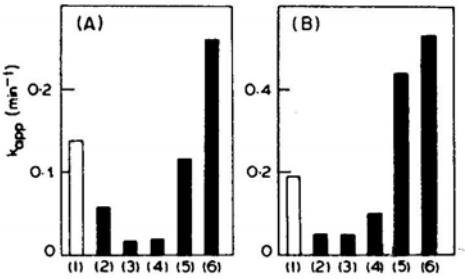

Figure 12. Summary of the pseudo first order rate constants for the inactivation of the enzyme. **A.** NEM (0·4 mM).
B. Phenylglyoxal (10 mM) (1) Control, (2) + 0·1 mM Mn^{2+}, (3) + 10:20 mM ATP:Mg^{2+}, (4) +10:10mM ATP:Mg^{2+} (5) +10:5mM ATP:Mg^{2+} and (6) + 10 mM ATP.

the enzyme against inactivation (decreased k_{app} values), free ATP enhanced the rate of inactivation (increased k_{app} values) in both the cases.

Discussion

The regulation of glutamine synthetase levels in *A. niger* appeared to be different from that observed in other fungi. In the case of *A. nidulans, Neurospora crassa* and *Candida utilis,* only Glu specifically elevated the levels of the enzyme, whereas in *A. niger* the enzyme levels were also enhanced by the presence of NO_3^- as the sole nitrogen source. One probable explanation for this observation could be the slow conversion of NO_3^- to NH_4^+ (Pateman *et al.,* 1967) and as the NHL concentrations did not reach high levels, glutamine synthetase activity was probably not repressed under these conditions. Repression of this enzyme levels by NH^+_4 was indicated by the results presented in table 2.

The near basal levels of glutamine synthetase observed when *A. niger* was grown in a medium containing low concentrations of an easily assimilable nitrogen source, such as Gln or NH_4^+ in addition to Glu or NO_3^- (table 1) suggested that Glu and NO_3^- are not specific inducers of this enzyme. A rational explanation for these observations is that the levels of glutamine synthetase respond reciprocally to the amount of easily

assimilable nitrogen supply. The fact that at low concentrations of NH_4^+, the enzyme levels are high (table 2), substantiate this suggestion. Further evidence in support of this contention was the observation that methylamine, which is a poor nitrogen source, enhanced the enzyme levels (table 1). The intensity of the protein stain (figure 2A) as well as the peak heights in the gel filtration profiles (figure 2) and the increased specific activity of glutamine synthetase present in the extracts, suggested that there was a larger amount of enzyme protein in the Glu and NO_3^- grown cells, compared to NH_4^+ grown cells. These results are fully compatible with the above hypothesis.

In addition to regulation by NH_4^+ repression, bacterial glutamine synthetase is also known to be modulated by covalent modification (Stadtman and Ginsburg, 1974) and the fungal enzyme by association-dissociation depending on the nitrogen status (Vichido *et al.,* 1978; Sims *et al.,* 1974b), as well as, by specific rapid inactivation (Ferguson and Sims, 1974b; Legrain *et al.,* 1982; Van Andel and Brown, 1977). These mechanisms do not appear to be operating in the case of *A. niger* glutamine synthetase. The absence of regulation by covalent modification was indicated by the constant ratio of Mg^{2+}-dependent synthetase to Mn^{2+}-dependent *y*-glutamyl transferase activity, a criterion employed to indicate this type of regulation (Pateman, 1969; Sims *et al.,* 1974b). However, the identical elution profiles (figure 2) and electrophoretic mobility (figure 2A) under different growth conditions clearly indicated the absence of association-dissociation as a method of regulation of the enzyme in *A. niger*. Yeast glutamine synthetase was rapidly inactivated when a preferred nitrogen source *e.g.,* NH_4^+ or glutamine was added to the exponentially growing cells (Ferguson and Sims, 1974b; Legrain *et al.,* 1982; Van Andel and Brown, 1977). Such a mechanism may not be operating in *A. niger,* as NH_4^+ and glutamine caused only a slow and marginal decrease in the activity (figure 3). The absence of rapid inactivating system in this fungus was also borne out by the fact that glutamine synthetase was not inactivated on mixing the crude extracts obtained from cells grown on different nitrogen sources (data not given). All these observations point to the differences in the regulatory mechanisms that operate in the control of this enzyme activity in different fungi.

Sporulation in *A. niger* was considerably delayed when grown on 100 mM NO_3^- (120 h compared to 72 h, figure 1) Rapid loss of glutamine synthetase activity just prior to sporulation suggested that, glutamine synthetase and hence the nitrogen status (Galbraith and Smith, 1969) Could be a signal for sporulation in this fungus. Such a correlation between sporulation and glutamine metabolism has been observed in the case of *Bacillus subtilis* (Bott *et al.,* 1977).

To further our studies on the molecular mechanisms of the regulation of glutamine synthetase, the enzyme from *A. niger* was purified to apparent homogeneity. The *A. niger* enzyme did not bind to many affinity matrices used in the purification of this enzyme (Seethalakshmi and Appaji Rao, 1979; Iyer *et al.,* 1981; Lepo *et al.,* 1979; Mitchell and Magasanik, 1983; Tuli and Thomas, 1981; Palacios, 1976). The enzyme was weakly adsorbed on to Glu-Sepharose (Lin and Kapoor, 1978), probably by ionic interactions rather than by specific bioaffinity. These observations again highlight the differences in the properties of *A. niger* glutamine synthetase compared to the enzyme from other sources.

The M_r of the native glutamine synthetase from *A. niger* (385,000±25,000) was similar to the enzyme reported from other fungal sources (Sims *et al.,* 1974a; Palacios,

1976). From the SDS-polyacrylamide gel electrophoresis of the enzyme it was established that the *A. niger* enzyme was octameric and was composed of identical subunits. In this respect, glutamine synthetase from *A. niger* resembles other fungal and mammalian enzymes (Stahl and Jaenicke, 1972; Tate and Meister, 1973). Glycerol (5%) effectively stabilized the enzyme against inactivation during storage and also against repeated freezing and thawing.

The characteristic influence of the metal ion activators on the pH optimum of the enzyme (figure 5) may have some physiological significance for this organism as it excretes citric acid under acidic pH conditions and Mn^{2+} deficiency (Kubicek and Rohr, 1978). Intracellular pH and the ratio of Mg^{2+} to Mn^{2+} inside the cell could be regulating the glutamine synthetase activity.

Like glutamine synthetase from all the other sources, *A. niger* enzyme also exhibited hyperbolic saturation patterns with Glu, Mg. ATP and NH_2OH (or NH_4^+). A plot of pK_m against pH, indicated that the binding of Glu to the enzyme required an ionizable group with a pK_a of 6·5 probably a histidine (figure 6, Inset). Such a pH dependent Glu saturation was reported for mammalian glutamine synthetase (Schnackerz and Jaenicke, 1966). None of the ionizable groups of the substrates titrate with a pK_a value of 6·5. The high K_m value for Gln (235 mM), may account for the rapid breakdown of [E. glutamine] complex thereby making the biosynthetic reaction of *A. niger* glutamine synthetase essentially irreversible.

The availability of highly purified *A. niger* glutamine synthetase in reasonable amounts, permitted a critical evaluation of the essential amino acid residues required for activity. Arginine and cysteine have been implicated as active site amino acids in glutamine synthetases (Shapiro and Stadtman, 1967; Uralets *et al.,* 1977; Rao *et al.,* 1973; Jaenicke and Berson, 1977; Powers and Riordan, 1975). Phenylglyoxal was used to locate reactive arginine residues in the *A. niger* glutamine synthetase. From the inactivation data, replot of the pseudo first order plots (figure 7) suggested that an arginine residue on the enzyme was probably reacting with 2 mol of phenylglyoxal. Such a reaction has been observed with active site arginine residues in several enzymes (Riordan, 1979). Of the three substrates, only Mg. ATP complex protected the enzyme against inactivation, in a concentration dependent manner and the constant for protection calculated (0·9 mM, figure 9A) was comparable to the K_m value of Mg. ATP complex (1·5 mM, table 4). These results would suggest that the reactive arginine residue may be the Mg. ATP binding subsite at the active site. This was further substantiated by the fact that Mg. ADP and Mg. AMP which are competitive inhibitors of the enzyme with respect to Mg. ATP (data not shown), also protected it against phenylglyoxal inactivation. The observation that k_{app} values for the three ligand complexes followed the order, Mg. AMP > Mg.ADP > Mg.ATP, also point to the occurrence of arginine at the active site. If the interaction of the nucleotide with arginine involves the phosphate group(s) of the nucleotide, it is to be expected that the reduction in the number of phosphates would decrease the strength of interaction.

Almost all the glutamine synthetases studied so far are thiol proteins, as shown by the decrease of enzyme activity by a variety of thiol inhibitors *e.g., p*-hydroxymercuribenzoate (*p*HMB), NEM, iodoacetate and 5,5-dithio-*bi*s-2-nitrobenzoic acid. The results using NEM (figure 10A and B) clearly point out that a reactive cysteine may be essential for the activity of *A. niger* glutamine synthetase.

Low concentrations of manganese protected the enzyme against inactivation by both phenylglyoxal and NEM (figures 9B and 11). Based on the concentration dependence of this protection, the dissociation constants of 52 μM and 14 μM for Mn^{2+} were calculated (which are in reasonably close agreement, taking into account the limitations of the methods used). This data also suggested the presence of a single high affinity Mn^{2+} binding site on the enzyme. The kinetic constant for Mn^{2+} obtained from steady state kinetic analysis (Punekar, 1983) was atleast an order of magnitude higher than the binding constants obtained from protection experiments, indicating the presence of more than one class of Mn^{2+} binding sites on the enzyme. In the case of *E. coli* glutamine synthetase, based on the protection experiments, it was demonstrated that Mn^{2+} binding at the high affinity site protected the enzyme against inactivation reflecting the stability of the 'taut' form of the enzyme (Shapiro and Stadtman, 1967). It is evident that like in the glutamine synthetases from other sources (Hunt and Ginsburg, 1980; Shapiro and Stadtman, 1967; Uralets *et al.,* 1977; Jaenicke and Berson, 1977). *A. niger* enzyme also probably requires a histidine, arginine and a sulphydryl group for catalytic activity.

Although *A, niger* enzyme is regulated differently from the enzyme from other bacterial and fungal sources, the active site residues seem to be well conserved.

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