Role of Mn^{2+} and Mg^{2+} in Catalysis and Regulation of *Aspergillus niger* Glutamine Synthetase

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The kinetic data on the effect of Mn^{2+}, Mg^{2+} and ATP on the *Aspergillus niger* glutamine synthetase activity analysed according to the isovelocity method [London W P & Steck T L (1969) *Biochemistry*, 8, 1767] revealed that the enzyme under physiological conditions is probably an Mn(II) enzyme. Excess of ATP or Mn^{2+} beyond the concentrations required to form the metal ion-nucleotide complex inhibited both the Mg^{2+} and Mn^{2+} supported glutamine synthetase activity, whereas excess Mg^{2+} was not inhibitory. These results along with the inhibition of enzyme activity by Ca^{2+}, Zn^{2+}, Co^{2+}, GTP and EDTA indicated that the interactions of the enzyme with Mn^{2+} and Mg^{2+} were different. Supporting evidence for the proposed kinetic mechanism was obtained by the protection afforded by low concentrations of Mn^{2+} ($<100\,\mu M$) against inactivation of the enzyme by N-ethylmaleimide or phenylglyoxal. However, ATP enhanced the rates of inactivation by both modifying agents. K_{Mn}, values of 52 and 14 μM calculated from the protection afforded by this metal ion against inactivation by phenylglyoxal and N-ethylmaleimide suggested the presence of a high affinity Mn^{2+} binding site on the enzyme, in addition to the binding of Mn^{2+} ATP complex at the active site. These results permit the conclusion that *A. niger* glutamine synthetase may be an Mn(II) dependent enzyme under physiological conditions and the metal ion in addition to serving as a substrate when complexed with ATP may have an additional role in protecting the enzyme against inactivation.

Glutamine synthetase (L-glutamate: ammonia ligase, EC 6.3.1.2) catalyzes the formation of glutamine, a precursor for the synthesis of a variety of nitrogenous end products. The activity and content of this enzyme located at a crucial point in the highly branched pathway of nitrogen metabolism, is regulated particularly in microorganisms by alterations in the growth medium, by accumulated end products and by availability and nature of divalent metal ion activator[1-4]. A derangement of carbohydrate metabolism leading to excretion of citric acid into the medium by *Aspergillus niger* is extensively used for the production of this compound by fermentation. The conditions of fermentation, viz. low pH, high amounts of carbon source and essentially Mn^{2+}-deficient medium[3], resulting in elevated intracellular NH_4^+ pools[6], accumulation of L-glutamate, L-glutamine and amino acids derived from it[7] suggested to us that the metabolic interlock between carbon and nitrogen metabolism may have been deranged at the glutamine synthetase step. Although at first glance the increase in glutamine levels[6,7] appears to be not in agreement with the above hypothesis, a closer examination of their data, especially when ratios of L-glutamate or L-

glutamine *vs* time and the amounts of amino acids derived from glutamate are plotted, reveals that the decrease in glutamine synthetase flux becomes significant during citric acid fermentation. Our earlier work revealed that the activity of glutamine synthetase from *A. niger* was not regulated by covalent modification, specific proteolytic inactivation or by effective feedback inhibition[8,9]. However, as citric acid excretion occurred when the concentration of Mn^{2+} in the medium was very low and as one of the biosynthetic activities of glutamine synthetase required manganese ions, it was of interest to examine the role of Mn^{2+} and Mg^{2+} ions in the catalysis and regulation of the homogeneous glutamine synthetase isolated from *A. niger*[9]. This paper describes kinetic analysis to indicate the possible presence of a tight binding site for Mn^{2+} and a relatively weak metal ion binding site on the enzyme where Mn^{2+} or Mg^{2+} nucleotide complexes can bind as substrates.

Experimental Procedures

Reagents including ATP, sodium L-glutamate, hydroxylamine hydrochloride, GTP, imidazole, phenylglyoxal and N-ethylmaleimide (NEM) were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. All other reagents were of the analytical grade. ATP, GTP and EDTA were neutralized to pH 7.0 with 2 M NaOH.

Enzyme assays —Glutamine synthetase from *A. niger* was purified to homogeneity, as determined by polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulphate (SDS) - PAGE, by

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Abbreviations used: NEM, *N* ethylmaleimide; DEAE-, diethylaminoethyl-; K, dissociation constants from either kinetic or protection experiments; k, inactivation rate constant; γ-GHA, γ-glutamylhydroxamate; E, free enzyme; M, metal ion (Mn^{2+} or Mg^{2+}); PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate.

ammonium sulphate fractionation, diethylaminoethyl (DEAE) - sephacel chromatography, AMP-sepharose affinity chromatography and gel filtration on Sepharose 4B[8,9].

Glutamine synthetase activity was determined by estimating γ-glutamylhydroxamate (γ-GHA) as described elsewhere[9,10].

Protein was estimated according to the method of Lowry *et al.*[11], using bovine serum albumin as the standard.

One unit of enzyme activity is defined as the amount of enzyme required to produce one μmole or γ-GHA per min at 28°C.

Chemical modifications — *A. niger* glutamine synthetase was inactivated completely on incubating with phenylglyoxal or NEM[9]. For monitoring the inactivation of the enzyme by NEM, it was passed through a G-25 column (1 × 5 cm) just before use to remove 2-mercaptoethanol added to stabilize the enzyme. The inactivation of the enzyme by either phenylglyoxal (10 mM) or NEM (0.4 mM) was carried out at 28°C by incubating the enzyme (40-60 μg) in a scaled up reaction mixture (500 μl) and with drawing aliquots (50 μl) at different time intervals indicated in the legends to the figures. The reaction was terminated by diluting the enzyme directly into assay mixtures which did not contain the inactivating agent. 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 agent, was normalized to 100 and the residual activity after different periods of inactivation was expressed as per cent of this normalized value. There was a parallel loss of both Mg^{2+} dependent synthetase and Mn^{2+} dependent transferase activity, when the enzyme was inactivated by these reagents. Similarly, the protection afforded by the various ligands against inactivation of the enzyme by the two reagents was identical for both the enzyme activities and was monitored by including the compounds in the inactivating system at appropriate concentrations. hence γ-glutamyl-transferase activity values alone are reported to represent inactivation data. Phenylglyoxal interfered with the colorimetric assay[10] and appropriate controls were run to circumvent this problem.

Binding constants were estimated by the ability of Mn^{2+} or Mg^{2+}-ATP complex to protect the enzyme against inactivation by NEM or phenylglyoxal[12,13] using the following equation:

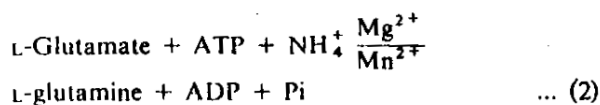$$\ln \frac{k_o - k_p}{k_p} = n \ln [P] - \ln K_p \qquad \qquad \dots (1)$$

where k_o represents the rate constant of inactivation in the absence of protective ligand P, k_p, the rate constant of inactivation in the presence of ligand P and [P], the molar concentration of the ligand. A plot of $\ln[(k_o - k_p)/k_p]$ against $\ln[P]$ yields a straight line whose slope indicates the number of molecules of P bound per active site and the ordinate intercept ($-\ln K_p$) provides the dissociation constant, K_p for the E-P complex.

Analysis of data — The isovelocity method and other analytical replots suggested by London and Steck[14] were employed to analyze the activity profiles of both Mn^{2+} and Mg^{2+} supported glutamine synthetase activity.

The kinetic data were analyzed by least-square curve fitting procedures (linear regression) using a programmable pocket calculator (Texas Instruments SR-51A).

Results

Interaction of A. niger glutamine synthetase with divalent cations — The biosynthetic reaction (Eq.2) of *A. niger* glutamine synthetase required either Mg^{2+} or Mn^{2+} as metal ion activator

$$\text{L-Glutamate} + \text{ATP} + \text{NH}_4^+ \xrightarrow{\underset{\text{Mn}^{2+}}{\text{Mg}^{2+}}}$$

$$\text{L-glutamine} + \text{ADP} + \text{Pi} \qquad \qquad \dots (2)$$

and ATP as the nucleotide substrate. Several divalent metal ions such as Ca^{2+}, Ba^{2+}, Sr^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+} and Cr^{2+} could not replace either Mg^{2+} or Mn^{2+} (ref.9), although many of these complex with ATP[9]. The study of the effect of GTP, EDTA and divalent metal ions on the Mn^{2+}-dependent synthetase activity showed that while excess Mn^{2+} inhibited the enzyme activity, excess Mg^{2+} was without any effect (Table 1). Also, Ca^{2+} and Zn^{2+} inhibited the enzyme reaction at all concentrations of Mn^{2+} studied. Cobalt (II), however, was without any effect at the optimal Mn^{2+} concentration but caused a very significant activation at a low concentration of Mn^{2+} (1 mM) and less marked activation at excess Mn^{2+} (10 mM). GTP inhibited the enzyme activity at 1 and 4 mM Mn^{2+} and at 5 and 10 mM Mg^{2+}. Although the effect of EDTA was qualitatively similar to that of GTP, it was quantitatively more pronounced.

The concentration of Mg^{2+} beyond 20 mM had no significant effect on the Mg^{2+} dependent synthetase activity (Fig 1A and Table 1). Ca^{2+} and Zn^{2+} very specifically inhibited the Mg^{2+} supported biosynthetic reaction at all concentrations of Mg^{2+}. Cobalt, on the other hand, enhanced the activity at the lowest concentration of Mg^{2+} (5 mM), and was without effect at higher concentrations of Mg^{2+}. It is interesting to note that Co^{2+}, Zn^{2+} and Cd^{2+} do not substitute for

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Table 1—Effect of GTP, EDTA and Divalent Metal Ions on the *A. niger* Glutamine Synthetase Activity

Addition (mM)	Activity (%)[a]					
	Mn^{2+} (mM)			Mg^{2+} (mM)		
	1	4	10	5	10	20
None	18	100[b]	45	6	95	100[c]
+Ca^{2+} (10)	9	24	29	0	1	3
+Zn^{2+} (10)	8	11	12	1	2	1
+Co^{2+} (10)	107	102	77	67	90	82
+GTP (5)	4	62	71	2	47	92
+EDTA (5)	0	6	101	0	76	105

[a]The reaction mixtures contained 100 mM imidazole hydrochloride buffer pH 7.8 for Mg^{2+}-dependent synthetase activity and pH 5.5 for Mn^{2+}-dependent synthetase activity, 50 mM NH$_2$OH, 100 mM L-glutamate, 10 mM ATP and various additions at concentrations shown in the table. The reaction was started by the addition of enzyme.

[b]The Mn^{2+}-dependent synthetase activity (pH 5.5) at optimal concentration of Mn^{2+}(4 mM) was normalized to 100 and the other numbers are per cent of this normalized value.

[c]The Mg^{2+}-dependent synthetase activity (pH 7.8) at optimal concentration of Mg^{2+} (20 mM) was normalized to 100 and the other values are expressed as per cent of this activity.
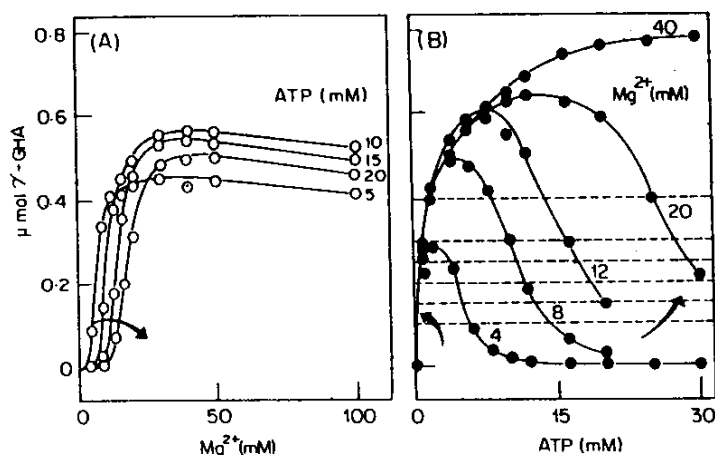

Fig. 1—Saturation of *A. niger* Mg^{2+}-dependent glutamine synthetase activity by Mg^{2+} and ATP [(A), Mg^{2+}-profiles. The reaction mixture (0.5 ml) contained 100 mM imidazole hydrochloride buffer (pH 7.8), 50 mM NH$_2$OH and 100 mM L-glutamate. MgCl$_2$ concentration was varied in the range 0-1(0 mM at 5,10,15 and 20 mM ATP. The reaction was started by the addition of enzyme (9.6 μg). (B), ATP-profiles. The enzyme assays were carried out in the same manner as described above with ATP concentration varied in the range 0-30 mM at 4,8,12,20 and 40 mM MgCl$_2$. Broken horizontal lines indicate the isovelocity points used for the replots in Fig.2B. Arrows indicate the change in sigmoidicity with increasing fixed concentrations of the ligand] .

Mn^{2+} or Mg^{2+} as metal activators. It is also clear from Table 1 that GTP inhibited the activity at 5 and 10 mM Mg^{2+}. The effect of EDTA was similar to that observed with GTP.

Increasing concentrations of Mn^{2+} ions progressively inhibited the Mg^{2+}-supported glutamine synthetase reaction, with 0.2 mM Mn^{2+} causing 50% inhibition. In view of these differences and complexity in the pattern of interaction of glutamine synthetase with Mg^{2+} and Mn^{2+}, the effect of these metal ions was examined in more detail.

In several experiments, the enzyme was preincubated with either the divalent metal ion (Mg^{2+} or

Mn^{2+}) or the nucleotide (ATP) before starting the reaction by the addition of one of the substrates. Also, in the case of *E. coli* glutamine synthetase, Mn^{2+} converts the enzyme into a "taut" form and a lag in the time course of the synthetase reaction is observed[16]. It was therefore necessary to establish that preincubation of *A. niger* enzyme with these ligands did not cause either a lag or burst in the initial velocity of the reaction. When the glutamine synthetase reaction was started by the addition of enzyme, ATP, NH$_2$OH or metal ion, identical and overlapping patterns of initial velocity were obtained (data not presented).

Kinetics of Mg^{2+} activation—The saturation of *A.*

niger enzyme with Mg^{2+} at different fixed concentrations of ATP (termed Mg^{2+}-profiles) as well as the pattern of ATP saturation at different fixed concentrations of Mg^{2+} (termed ATP-profiles) was determined. It can be seen from Fig. 1A that all the Mg^{2+}-profiles were sigmoid and their sigmonidicity was dependent on the concentration of ATP present (increasing sigmoidicity with increasing fixed concentration of ATP). Also, there was no inhibition at higher concentrations of Mg^{2+}. Maximal activity was obtained when Mg^{2+} and ATP ratio was about 2:1. The marginal inhibition (5-10%) at the highest concentration of Mg^{2+} tried was not significant and was probably due to changes in the ionic strength.

There was no apparent sigmoidicity in the ascending limbs of ATP-profiles (Fig.1B) but after the peak of activity was reached, increasing concentrations of ATP caused progressively larger inhibition of enzyme activity. The maximal velocity in each case was different and was dependent on the fixed concentration of Mg^{2+}. The peak positions were at Mg^{2+}: ATP ratio of approx. 2:1. When Mg^{2+} was present in excess (40 mM) over ATP (10 mM), the ATP-profile was hyperbolic. A K_m value of 1.5 mM was obtained for Mg^{2+}-ATP complex from a double-reciprocal plot.

The Dixon-plots for inhibition by ATP were drawn (Fig.2A) using the data from the descending limbs of ATP-profiles (Fig.1B). The plot shown in Fig.2A is nonlinear (parabolic) indicating multiple interacting sites for ATP on the enzyme. The nonlinearity of the Dixon-plots precluded the determination of the K_i value for free ATP from these curves. Hence it was obtained from the isovelocity replot of the data
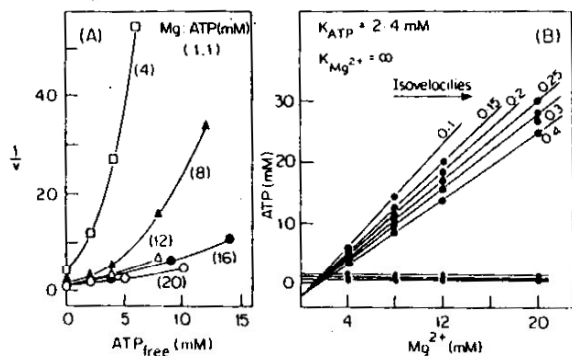
presented in Fig.1B (broken horizontal lines). At each isovelocity point, the two concentrations of ATP for each Mg^{2+} concentration were obtained by extrapolation. These two sets of extrapolated ATP concentrations were plotted against the corresponding concentration of Mg^{2+} according to London and Steck[14]. At low ATP concentrations (Mg^{2+} excess region), the isovelocity replots (Fig.2B) were linear and parallel to Mg^{2+}-axis. On the other hand, at higher ATP concentrations, a set of intersecting lines (Fig.2B) was obtained and the point of intersection on the ATP axis gave a K_{ATP} value of 2.4 mM.

Kinetics of Mn^{2+} activation—The velocity profiles for Mn^{2+}-dependent synthetase activity were studied at its pH optimum of 5.5. The Mn^{2+}-profiles (Fig.3A) were bell shaped with sigmoid ascending limbs. The position of the peak velocity was dependent upon the fixed concentration of ATP and maxima were reached
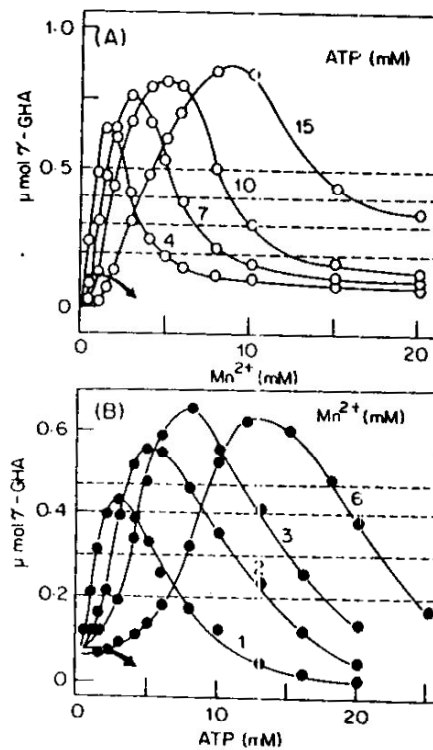

Fig. 3—Mn^{2+} and ATP-saturations of A. niger Mn^{2+} dependent glutamine synthetase activity [(A), Mn^{2+}-profiles. The reaction mixture (0.5 ml) contained 100 mM imidazole hydrochloride buffer (pH 5.5), 50 mM NH_2OH and 100 mM L-glutamate, $MnSO_4$ concentration was varied in the range 0-20 mM at 4,7,10 and 15 mM ATP. The reaction was started by the addition of enzyme (16 μg). (B), ATP-profiles. The reaction mixtures were same as described above except that ATP concentrations were varied in the range 0-25 mM at 1,2,3 and 6 mM $MnSO_4$. Arrows indicate the shift of curves with increasing fixed concentrations of the ligand. Broken horizontal lines represent the isovelocities used in the replots shown in Fig.4]

Fig. 2—Kinetics of inhibition of A. niger Mg^{2+}-dependent glutamine synthetase activity by ATP [(A), Dixon plot for ATP inhibition. Replot of data from the descending limbs of ATP saturation (Fig.1B). The free ATP concentration was computed as follows: $[ATP]_{free} = [ATP]_{total} - [Mg^{2+}]_{total}$ (eqn.7) and the 1:1 complex of Mg·ATP was assumed to be the true substrate. (B). Isovelocity replot of ATP profiles. The isovelocity data from Fig.1B (broken horizontal lines) were used and the two concentrations of ATP at each $MgCl_2$ concentration were plotted as coordinate axes]

at Mn^{2+} :ATP ratio of about 1:2. The sigmoidicity of the ascending limbs increased with increasing fixed concentration of ATP (arrow in Fig.3A). It is interesting to note that unlike Mg^{2+}-profiles (Fig.1A), the Mn^{2+}-profiles showed a distinct inhibitory phase.

The ATP-profiles for the Mn^{2+}-supported synthetase assay are shown in Fig.3B. The velocity profiles were once again bell shaped with sigmoid ascending limbs. The sigmoidicity was more apparent at higher fixed concentrations of Mn^{2+} and the peak velocity occurred when Mn^{2+} :ATP ratio was about 1:2.

The constants K_{ATP}, $K_{Mn^{2+}}$ and $K_{Mn.ATP}$ were obtained by isovelocity analysis[14] of the data. The two concentrations, of Mn^{2+} corresponding to a fixed concentration of ATP at each isovelocity point (broken horizontal lines in Fig.3A) from Mn^{2+}-profiles were plotted. In this replot, a family of lines, defined by the equation,

$$V[ATP]_{total} + v.K_{ATP} -$$

$$[v+(V-v)\frac{K_{ATP}}{K_{Mn^{2+}-ATP}}] [Mn^{2+}]_{total} = 0 \qquad ... (3)$$

(where v is the isovelocity and V is the maximal

velocity) for the ATP excess region ([ATP] >>[Mn^{2+}]) intersect on ATP-axis to give K_{ATP} value of 1.2 mM (Fig. 4A). On the other hand at high Mn^{2+} concentration (Mn^{2+} excess region [Mn^{2+}] >>[ATP]), the curves follow the equation.

$$v[Mn^{2+}]_{total} + v.K_{Mn^{2+}}$$

$$-[v+(V-v) \frac{K_{Mn^{2+}}}{K_{Mn^{2+}-ATP}}] [ATP]_{total} = 0 \qquad ... (4)$$

and intersect on the Mn^{2+} axis at a $K_{Mn^{2+}}$ value of 0.7 mM. A similar analysis of ATP-profiles using points of equal velocity (broken horizontal lines in Fig.3B) gave K_{ATP} and $K_{Mn^{2+}}$ values of 1.0 mM and 0.5 mM respectively (Fig.4B). The constants obtained for Mn^{2+} and ATP from ATP- and Mn^{2+}-profiles were similar and an average of the two values is presented (Table 2). The slopes of the asymptotically linear portions of the isovelocity replots (Fig.4A and B) in the Mn^{2+} excess region is given by,

$$\frac{d[Mn^{2+}]}{d[ATP]} = 1 - \frac{K_{Mn^{2+}}}{K_{Mn^{2+}-ATP}} + V \frac{K_{Mn^{2+}}}{K_{Mn^{2+}-ATP}} \cdot 1/u.. \quad (5)$$
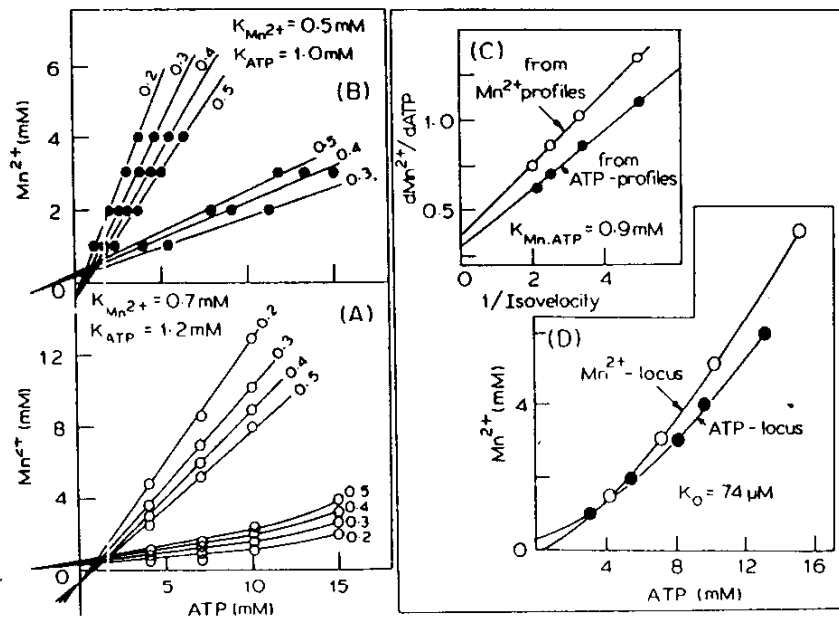
Therefore these slopes when replotted against the

Fig. 4—Replots for the determination of $K_{Mn^{2+}}$, K_{ATP}, $K_{Mn^{2+}-ATP}$ and K_0 for the Mn^{2+}-dependent synthetase reaction [(A). The plot of the two concentrations of Mn^{2+} corresponding to a fixed concentration of ATP at the isovelocities indicated by broken lines in Fig.3A.(B), a plot of the two concentrations of ATP corresponding to a fixed concentration of Mn^{2+} at the isovelocities indicated by broken lines in Fig.3B. (C), a plot of the slopes (d Mn^{2+}/d ATP) of the asymptotically linear portion of the isovelocity replots in the Mn^{2+} excess region (Fig.4A and B) against the reciprocal of corresponding isovelocities from Mn^{2+}-profiles (O) and ATP-profiles (●). (D), locus of peak velocities. A plot of the concentration of Mn^{2+} and ATP at the peak velocity for each of the ATP-profiles (●) and Mn^{2+}-profiles(O)]

reciprocals of corresponding isovelocities gave a straight line (Fig.4C) and from the Y-axis intercept $(=1-\frac{K_{Mn^{2+}}}{K_{Mn^{2+}\text{-}ATP}})$, the value of $K_{Mn^{2+}\text{-}ATP}$ was calculated to be 0.9 mM. This $K_{Mn^{2+}\text{-}ATP}$ value is an average of the values obtained from the analysis of Mn^{2+} excess region of ATP-profiles ($=0.86$ mM) and Mn^{2+}-profiles ($=0.95$ mM). Only data from Mn^{2+} excess region ($[Mn^{2+}]>>[ATP]$) were used as they were more accurate.

The concentrations of Mn^{2+} and ATP at the peak velocity for each of the ATP-profiles (Fig.3B) and Mn^{2+}-profiles (Fig.3A) were plotted as coordinate axes (called 'locus of the peak velocities'). Such an analysis of ATP-profiles (intercept on ATP-axis, K_oK_{ATP},Fig. 4D) and Mn^{2+}-profiles (intercept on $Mn^{2\pm}$ axis, $K_oK_{Mn^{2+}}$, Fig. 4D) gave K_o value of 74 μM (Table 2) by substituting K_{ATP} and $K_{Mn^{2+}}$, independently determined in this study. A summary of the kinetic constants is given in Table 2.

Velocity profile at equimolar ratios of ATP and Mn^{2+} but at different concentrations was sigmoidal with half-maximal saturation at 18 mM (Fig.5).

Determination of binding constants for Mg^{2+}-ATP complex and Mn^{2+} by their ability to protect against inactivation of the enzyme by phenylglyoxal and NEM — A. niger glutamine synthetase was completely inactivated by reaction with two moles of
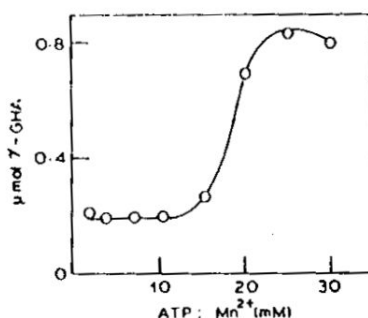

Fig. 5—Effect of varying ATP and Mn^{2+} concentration at equimolar ratio [The enzyme assays were performed in the same manner as described in the legend to Fig.3A except that the reaction mixtures contained different amounts of ATP and Mn^{2+} but in equimolar concentrations. Twelve μg of the enzyme was used per 0.5 ml of the assay mixture]

phenylglyoxal or by one mole of NEM. The second order rate constants (at 28°C and pH 7.5) were determined to be 3.8 M^{-1} min^{-1} and 760 M^{-1} min^{-1}; for phenylglyoxal and NEM respectively[9]. In order to evaluate the binding constant for Mg^{2+}-ATP complex, the inactivation of the enzyme by phenylglyoxal was carried out in the presence of increasing concentrations of this ligand. The protection by Mg^{2+}-ATP complex was reflected by decreased pseudo-first order rates of inactivation (k_{app} values, inactivation profiles and first order plots are not shown). A replot of this data shown in Fig.6 gave a $K_{Mg^{2+}\text{-}ATP}$ (dissociation constant for E.Mg^{2+}-ATP complex) of 0.9 mM. A similar experiment for Mn^{2+}-ATP dependent synthetase activity (pH 5.5) was different from the pH for the inactivation (pH 7.5).

The K_{app} values for phenylglyoxal inactivation in the presence of increasing ATP:Mg^{2+} ratio followed a sigmoid pattern (Fig.7). Decreasing this ratio below 1:1 enhanced the protection as reflected by the decreased k_{app} values. On the other hand, when the ratio was higher, i.e. when there was a larger amount of free ATP in the mixture, the rate constants for inactivation were considerably larger. The effect of different ratios of ATP:Mg^{2+} on NEM inactivation of the A. niger enzyme was similar (Table 3) and protection was maximal around ATP:Mg^{2+} ratio of 1:1 to 1:2. Like in the case of phenylglyoxal inactivation, free ATP enhanced the rate of inactivation of the enzyme by NEM.

Free ATP also increased the rate of inactivation by phenylglyoxal in a concentration-dependent manner (Table 4), suggesting that E.ATP form of the enzyme was much more susceptible to this modification.

The enzyme was protected by low concentrations of Mn^{2+} ions against both (phenylglyoxal and NEM) inactivations in a concentration dependent manner.

Table 2—Kinetic and Dissociation Constants for the Interaction of Metal Ions, Nucleotide (ATP) and Metal Nucleotide Complexes with the A. niger Glutamine Synthetase

Ligand	Kinetic constant[a] (mM)	Dissociation constant[b] (mM)
Mg^{2+}-dependent synthetase activity		
Mg^{2+}-ATP complex	1.5	0.9
ATP	2.4	
Mg^{2+}	x	
Mn^{2+}-dependent synthetase activity		
Mn^{2+}-ATP complex	0.9	
ATP	1.1	
Mn^{2+}	0.6	0.052, 0.014[c] $K_o = 0.074$[d]

[a]Kinetic constants were obtained from saturation plots.

[b]Dissociation constants were obtained by measuring the protection afforded by the ligands against inactivation of the enzyme by phenylglyoxal.

[c]From protection experiments using NEM as the inactivating agent.

[d]Dissociation constant for the dissociation of Mn^{2+}-ATP complex into Mn^{2+} and ATP was calculated from replots of locus of peak velocities.
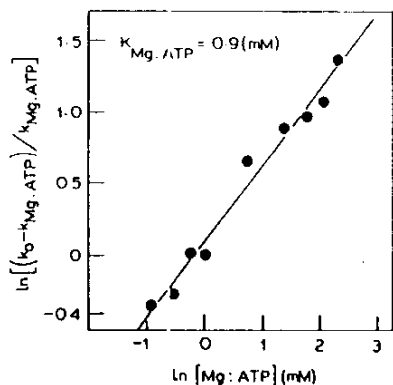
Fig. 6—The protection afforded to *A. niger* glutamine synthetase against phenylglyoxal inactivation by Mg^{2+}-ATP complex [The reaction mixtures containing the enzyme and different concentrations of Mg^{2+}:ATP (2:1 ratio and ATP varied between 0.4 to 10 mM) and 10 mM phenylglyoxal were incubated at pH 7.5, and at 28°C. Aliquots (50 µl) were withdrawn at 5 min intervals and the enzyme activity remaining was estimated by the Mn^{2+}-dependent γ-glutamyl transferase assay. From the time course of inactivation (not shown), the first order plots were constructed for the determination of rate constants (k_{app}) at the concentrations of Mg^{2+}-ATP used. A replot of this data as $\ln[k_0-k_{Mg^{2+}-ATP}/k_{Mg^{2+}-ATP}]$ against $\ln[Mg^{2+}-ATP]$ was used to evaluate the dissociation constant for the enzyme and Mg^{2+}-ATP complex]
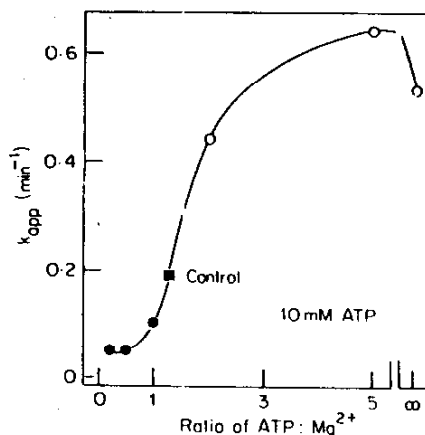

Fig. 7—Effect of different ratios of ATP[Mg^{2+}] on the k_{app} value for the inactivation of the enzyme by 10 mM phenylglyoxal. The inactivations were carried out in the same manner as described in Fig.6, and k_{app} values were calculated from pseudo-first order plots. ATP was used at 10 mM concentration with different concentrations of $MgCl_2$. (■). Control-10 mM phenylglyoxal alone: (●). protection: (O) enhanced inactivation]

From replots of k_{app} values for the phenylglyoxal inactivation at different Mn^{2+} concentrations and from similar experiments carried out with NEM, $K_{Mn^{2+}}$ values of 52 µM and 14 µM (Fig.8A&B) were obtained. The binding constants obtained from protection experiments are also listed in Table 2.

Table 3 Effect of Different Concentrations of ATP and $MgCl_2$ on the Inactivation of the Enzyme by NEM*

ATP (mM)	Mg^{2+} (mM)	k_{app} (min^{-1})
--	—	0.136
10	5	0.118
10	10	0.018
10	20	0.016
10	—	0.262

*Inactivation mixtures contained 50 mM imidazolehydrochloride buffer (pH 7.5), 0.4 mM NEM and enzyme (55 µg/500 µl) which was passed through a column of Sephadex G-25 to remove 2-mercaptoethanol, (For details, see Experimental Procedures) and $MgCl_2$ and ATP at concentrations indicated. Aliquots (50 µl) were withdrawn at 5 min intervals and the amount of active enzyme remaining was measured by Mn^{2+}-dependent γ-glutamyltransferase assay. k_{app} values were computed from the pseudo-first order plots

Table 4—Effect of ATP on the Inactivation of *A. niger* Glutamine Synthetase by Phenylglyoxal*

ATP (mM)	k_{app} (min^{-1})
0.0	0.029
0.1	0.041
0.5	0.057
0.7	0.064
1.0	0.083
2.0	0.162
4.0	0.217
6.0	0.281
8.0	0.351
10.0	0.393

*The inactivation of the enzyme by 4 mM phenylglyoxal was carried out at 28°C in 50 mM imidazole hydrochloride buffer (pH 7.5) and in the presence of concentrations of ATP shown in the table. Aliquots (50 µl) were withdrawn at 5 min intervals and the enzyme activity remaining was measured by Mn^{2+}-dependent γ-glutamyl transferase assay. k_{app} values were calculated from the pseudo-first order plots.

Discussion

Glutamine synthetase from *A. niger* specifically required either Mn^{2+} or Mg^{2+}-ATP complex as substrate[9]. Although other divalent metal ions, e.g. Ca^{2+}, Zn^{2+} and Co^{2+}, ATP complexes were not substrates for the biosynthetic reaction, their importance and possibly physiologically significant role in the *in vivo* regulation of this enzyme activity is indicated by the results presented in Table 1. Inhibitory effect of these divalent metal ions (Table 1) as well as the inhibition by Mn^{2+} of the Mg^{2+}-supported synthetase activity suggested the probable existence of an additional metal ion binding site on the enzyme for Mn^{2+}.
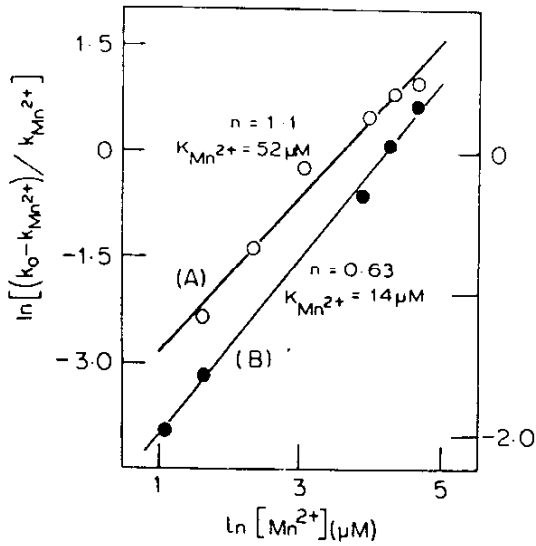
Fig. 8—Protection of A. niger glutamine synthetase against (A) phenylglyoxal and (B) NEM inactivation by Mn^{2+} ions [The inactivations were carried out as described in the legend for Fig.6 using 10 mM phenylglyoxal or 0.4 mM NEM but in the presence of 0-100 μM $MnSO_4$. From the k_{app} values obtained for both the inactivations at each Mn^{2+} concentration, $\ln[k_0-k_{Mn^{2+}}]/K_{Mn^{2+}}]$ versus $\ln[Mn^{2+}]$ was plotted. The enzyme activity was monitored by Mn^{2+}-dependent γ-glutamyl transferase assay. The enzyme was passed through Sephadex G-25 column to remove 2-mercaptoethanol prior to measuring the inactivation by NEM]

The effect of GTP on the A. niger glutamine synthetase activity was probably due to its metal complexing property and not due to feed-back inhibition as an end product of glutamine metabolism. The binding constant for ATP with Mg^{2+} or Mn^{2+} is comparable to that of GTP and hence it could shift the following equilibrium

$$ATP + Mg^{2+} \text{ (or } Mn^{2+}) \underset{\rightleftharpoons}{\overset{K_2}{}} Mg^{2+}\text{-ATP (or } Mn^{2+}\text{-ATP)} \quad \dots (6)$$
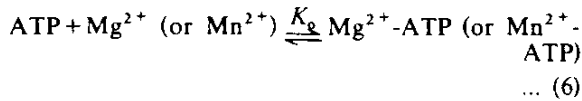
to the left resulting in a decrease in the effective concentration of the true substrate (metal-ATP complex) as well as an increase in the concentration of free ATP in the mixture. A combined effect of these would be a marked decrease in the velocity. This conclusion is supported by the observations that Mg^{2+} or Mn^{2+}-GTP complexes do not act as substrates. GTP is able to overcome inhibition caused by excess Mn^{2+} and GTP inhibition can be overcome by increasing the concentration of divalent metal ion (Mg^{2+} or Mn^{2+}). Further support for this conclusion was the observation that a well known metal chelator, EDTA, gave similar results, although a more pronounced inhibition was observed (Table 1).

The results obtained on the effects of Mg^{2+} and

Mn^{2+} on A. niger glutamine synthetase were analyzed using the criteria suggested by London and Steck[14] for metal activation namely: (a) nature of the ascending limbs of the velocity profiles;(b) relative position of the profiles obtained at higher fixed concentrations of ATP or metal ion;(c) the presence and position of peak velocity; (d) sigmodicity of the velocity profile with varied concentration but at equimolar ratio of metal ion and ATP; and (e) the velocity when metal ion or ATP was present in comparative excess.

Based on the kinetic experiments (Fig.1-5) and the protection afforded by Mn^{2+} against inactivation of the enzyme by phenylglyoxal and NEM (Figs 6 & 8) as well as our earlier observations[8,9], the general model proposed by London & Steck can be used to explain the interaction of Mn^{2+}, Mg^{2+} and ATP with A. niger glutamine synthetase (Fig.9). The forward triangle of the bottom face of the cube represents the equilibria for Mg^{2+}-supported activity and the entire bottom face represents the equilibria for the Mn^{2+}-dependent activity of A. niger glutamine synthetase. It should be emphasized at the outset that this mechanism does not make any assumptions regarding the order of binding of substrates or release of products. The observation with glutamine synthetase from different sources[17] suggests that a random kinetic mechanism may be operative. E (therefore represents free enzyme or enzyme complexed with either NH_2OH, L-glutamate or both) binds Mg^{2+}-ATP complex with a kinetic constant $K_{Mg^{2+}\text{-ATP}}$ (1.5 mM) to yield a productive enzyme substrate complex which turns over to yield free enzyme and products. E also binds free ATP (K_{ATP} = 2.4 mM) but not free Mg^{2+} in a kinetically significant manner as indicated by the absence of inhibition at excess Mg^{2+} and by the failure of this metal ion to afford protection against inactivation. As free ATP and Mg^{2+}-ATP complex compete for the same enzyme form, ATP is a competitive inhibitor with respect to Mg^{2+}-ATP.
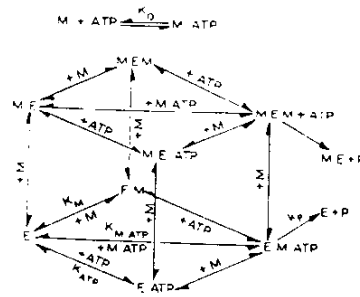

Fig. 9—Equilibria for the interaction of metal ion M (Mg^{2+} or Mn^{2+}) and ATP with A. niger glutamine synthetase based on the general model of London and Steck[14] for metal ion, nucleotide and enzyme interactions

Similar values of Michaelis constant for Mg^{2+}-ATP (1.5 mM) and the dissociation constant obtained from protection experiments (0.9 mM, Fig.6) suggested that binding at the active site was probably being monitored in both the cases. The sigmoid pattern of enhancement of enzyme activity when ATP-Mg^{2+} concentration was varied (Fig.1A) and protection against phenylglyoxal (Fig.7) and NEM (Table 3) inactivation are in support of the above suggestion.

Further support for the kinetic experiment suggesting that free ATP was binding to the enzyme was the observation that free ATP enhanced the rate of inactivation by phenylglyoxal (Table 4) or NEM (Table 3). Using modification by NEM it was demonstrated that mammalian glutamine synthetase was binding ATP and Mg-ATP complex at different sites[18].

In the case of Mn^{2+}-dependent glutamine synthetase activity, E reacts with Mn^{2+}-ATP complex with a kinetic constant $K_{Mn^{2+}-ATP}$ (0.9 mM) to form E.Mn^{2+}-ATP complex, which decomposes to form products and regenerates E. In addition, E also binds to Mn^{2+} or ATP separately with constants K_{Mn}. (0.6 mM) and K_{ATP} (11 mM) respectively. ATP reacts with free metal ion (Mn^{2+}) with a dissociation constant K_0 to yield the Mn^{2+}-ATP complex, which is the true substrate for the reaction. Hence it is evident that the velocity of Mn^{2+}-dependent synthetase reaction is dependent on the concentrations of Mn^{2+}-ATP and Mn^{2+}-ATP complex along with the four constants, namely, K_{Mn}, K_{ATP}, $K_{Mn^{2+}-ATP}$ and K_0 (Table 2). The inhibition by excess metal ion is characteristic of the Mn^{2+}-supported biosynthetic activity when compared to the Mg^{2+}-dependent reaction of *A. niger* glutamine synthetase. Additional evidence in support of Mn^{2+} interaction with the enzyme was the observation that GTP, which is not a substrate for the enzyme, relieved the inhibition caused by excess Mn^{2+} (Table 1) by complexing with it and making it unavailable for interaction.

Independent evidence for the interaction of Mn^{2+} with the enzyme (in addition to Mn^{2+}-ATP complex functioning as a substrate) was obtained by monitoring the protection afforded by low concentrations of Mn^{2+} against inactivation of the enzyme by NEM and phenylglyoxal (Fig.8). This Mn^{2+} protection and 'n' value of about one ($K_{Mn} = 52\ \mu M$ and $n = 1.1$, Fig.8A and $K_{Mn} = 14\ \mu M$ and $n = 0.63$, Fig. 8B) suggested the presence of at least one high affinity. Mn^{2+}-binding site on the enzyme. The kinetic constant for Mn^{2+} (0.6 mM) was an order of magnitude higher than the binding constant obtained from protection experiments, indicating that there are probably more than one class of Mn^{2+} binding sites on the enzyme. The presence of high affinity site for Mn^{2+} was also

suggested by the inhibition of Mg^{2+}-dependent synthetase activity by low concentrations of Mn^{2+}. A number of studies have shown that *Escherichia coli* glutamine synthetase has one tight binding, one intermediate binding and up to four weak metal ion binding sites per catalytic subunit[19-23]. Both tight binding (n_1) and intermediate binding (n_2) sites have been postulated to have a role in the catalytic activity of *E. coli* glutamine synthetase. However, in the case of *A. niger* enzyme, the n_1 sites represent the high affinity binding of free metal ion (Mn^{2+}) and n_2, the low affinity site, where Mn-ATP complex binds as one of the substrates. This mechanism is represented by the top face of the equilibrium cube (Fig.9). A recent report of a parallel study has led to the conclusion that glutamine synthetase from ovine brain may be a manganese (II) enzyme[3]. This enzyme, unlike glutamine synthetase from *A. niger*, shows high affinity kinetic interaction for both Mn^{2+} as well as Mg^{2+}, whereas the latter does not contain a kinetically meaningful high affinity site for Mg^{2+} ions.

The K_m values for Mn^{2+}-ATP and Mg^{2+}-ATP (Table 2) agree well with a recent estimate[24] of the intracellular concentration of ATP (1 mM) in *A. niger* in view of the high affinity (site) for Mn^{2+} (n_1 site) and the availability of sufficient amounts of Mn^{2+} within the *A. niger* cells[25], it could be postulated that *A. niger* enzyme occurs as a Mn^{2+}-protein. It has been suggested that Mn^{2+} ions can act as intracellular regulators[26,27]. The results presented here clearly indicate that glutamine synthetase could be a locus for regulation by Mn^{2+} ions.

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