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THE ROLE OF SPECIFIC CATIONS IN REGULATION OF CYANOBACTERIAL GLUTAMINE SYNTHETASE

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SUMMARY: Purified glutamine synthetase from the cyanobacterium *Anabaena cylindrica* required a divalent cation for activity. Maximum biosynthetic activity required Mg^{2+} (25 mM when supplied alone). Co^{2+} and Mn^{2+} each supported up to 20% of this activity; 12 other cations tested were ineffective. At 2.5 - 10 mM Mg^{2+}, 0.1 mM Co^{2+} or ethylene glycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid (EGTA) stimulated GS activity to maximum rates; other divalent cations (particularly Mn^{2+}) inhibited Mg^{2+}-dependent activity. At 5 mM Mg^{2+} the K_m^{app} for NH_4^+ (0.05 mM) was 20-fold lower than at 25 mM Mg^{2+}; added Co^{2+} did not markedly alter this low K_m for NH_4^+; this could be physiologically important.

INTRODUCTION: Glutamine synthetase (E.C.6.3.1.2) (GS), a key enzyme involved in NH_4^+ assimilation in cyanobacteria (see 1), requires ATP and a divalent cation for activity. We have previously purified and characterised GS from several cyanobacteria (2,3). Here we present information on the roles which Mg^{2+} and other divalent cations (especially Co^{2+}) may play in its activation and regulation.

MATERIALS AND METHODS

Organism and Growth Conditions: *Anabaena cylindrica* (CU 1403/2a) was grown axenically in batch culture as before (2).

Enzyme Extraction and Purification: GS was purified from *A. cylindrica* as before (2) but omitting aminohexane-Sepharose 4B chromatography and using instead sucrose gradient centrifugation. 1.5 ml aliquots of enzyme preparation were loaded onto continuous 0.2 - 0.8 M sucrose gradients in buffer B (50 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM sodium glutamate, 5 mM 2-mercaptoethanol and 1 mM EDTA). After centrifugation at 75,000 x g for 25 h at 4°C, active fractions were collected and could be stored at 4°C in 50% (v/v) glycerol in buffer B for several months without loss of activity.

Glutamine Synthetase Assay: All studies on the purified enzyme used the biosynthetic assay (4). Unless otherwise stated, the reaction mixture contained, in a volume of 1 ml: 1 μmol ATP; 25 μmol $MgCl_2$; 10 μmol sodium glutamate; 25 μmol NH_4Cl; 50 μmol Tris/HCl (pH 7.5) and 1-2 μg purified glutamine synthetase. In tests on the effects of cations on GS activity, aliquots of the enzyme stored in buffer B were desalted by chromatography on a 4 cm x 1 cm column of Sephadex G-25 and eluted in 50 mM Tris/HCl buffer (pH 7.5).

Protein Determination: The method of Lowry *et al.* (5) was used as before (2).

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Table. 1. The effects of various divalent cations on the Mg^{2+}-dependent activity of glutamine synthetase from *Anabaena cylindrica*

Concentration of cation (mM)	% of control activity										
	Ba^{2+}	Ca^{2+}	Cd^{2+}	Co^{2+}	Cu^{2+}	Fe^{2+}	Hg^{2+}	Mn^{2+}	Ni^{2+}	Sn^{2+}	Sr^{2+}	Zn^{2+}
0.05	99.8	76.0	97.3	108.0	95.8	98.2	41.1	43.6	100.0	94.6	75.2	94.9
0.5	96.6	25.4	13.2	107.0	84.5	33.3	29.5	6.1	91.6	3.9	24.0	70.8
5.0	55.2	2.7	2.7	58.0	49.0	12.6	14.3	2.8	39.0	0	5.8	5.7

Enzyme activities are percentages of the activity in the presence of 25 mM Mg^{2+} alone, which was 9.8 ± 0.2 μmol.P_i released.min^{-1}.(mg protein)$^{-1}$.

RESULTS AND DISCUSSION. Effects of various divalent cations on GS activity: Thirteen divalent cations (see Table 1) were tested alone, at concentrations up to 25 mM, for the effect of each on GS activity. Highest activity was obtained with 25 mM Mg^{2+} (9.8 μmol P_i released min^{-1} (mg protein)$^{-1}$); other cations supported negligible activity apart from Mn^{2+} and Co^{2+}. Table 1 shows the effects of the other cations tested on Mg^{2+}-dependent activity. Low Co^{2+} concentrations (0.05 - 0.5 mM) stimulated activity; other cations had no effect or were inhibitory. The optimum Mn^{2+} concentration (5 mM, at a Mn^{2+}:ATP molar ratio of 1:1), when added alone, supported 20% of maximum activity but at an optimum of pH 6.0, as in other organisms (6,7); the pH optimum in the presence of Mg^{2+} and/or Co^{2+} was 7.5. As the internal pH of cyanobacterial cells is 7.0 - 8.5 (8-10) Mn^{2+} alone, or in combination with Mg^{2+} (see below) may be of lesser importance *in vivo* than Mg^{2+} and Co^{2+} in supporting GS activity.

Stimulation of Mg^{2+}-dependent activity by added Co^{2+}. When Mg^{2+} was the only cation present (Fig. 1a), highest GS activity at optimum pH (7.5) and 1 mM ATP, required 25 mM Mg^{2+} (see also Table 1). However, the addition of 0.1 - 0.5 mM Co^{2+} stimulated GS activity at low Mg^{2+} (2.5 - 5 mM) to give virtually the same activity as that obtained with 25 mM Mg^{2+} alone. At high Mg^{2+} concentrations there was no enhancement of activity on adding Co^{2+} and Co^{2+} concentrations above 1 mM were inhibitory. The curves obtained when enzyme activity was plotted against Mg^{2+} concentrations were

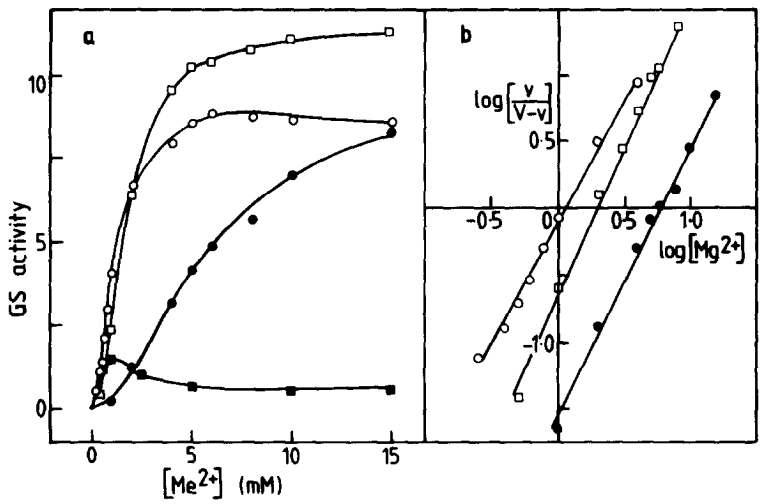

Fig. 1. Effects of Mg^{2+} and/or Co^{2+} on the biosynthetic activity of *Anabaena cylindrica* GS. (a), the effects of various Mg^{2+} concentrations in the presence (O) and absence (●) of 0.1 mM Co^{2+}, and in the presence of 0.1 mM EGTA (□), and of various Co^{2+} concentrations (■); (b) a Hill plot of the effects of various Mg^{2+} concentrations in the presence (O) and absence (●) of 0.1 mM Co^{2+} and in the presence of 0.1 mM EGTA (□). Activities are expressed as μmol.P_i released.min^{-1}.(mg protein)$^{-1}$.

sigmoidal and did not fit Michaelis-Menten kinetics; Hill plots (Fig. 1b) were indicative of positive co-operativity of Mg^{2+}-binding, whether or not Co^{2+} was present. The Mg^{2+} concentrations required for half maximum activity, calculated (11) from the data in Fig. 1b, were 6.9 mM and 1.2 mM in the absence and presence of Co^{2+}, respectively. Thus, within cyanobacterial cells, where the concentrations of Mg^{2+} alone are likely to be sub-optimal for GS activity, enhanced activity may be sustained if Co^{2+} is also available (see also below).

The role of Co^{2+} in stimulating GS activity at low Mg^{2+} concentrations:

Fig. 2 shows the effect on GS activity of varying the Co^{2+} concentrations at each of several fixed Mg^{2+} and ATP concentrations. Maximum stimulation of activity was obtained with 0.1 mM Co^{2+}, irrespective of the Mg^{2+} or ATP concentrations used. Thus, in these experiments, $MgATP^{2-}$ probably served as GS substrate and Co^{2+} appears to function as an activator. Our data do not support the view (12) that in the presence of Mg^{2+} $CoATP^{2-}$ serves as substrate with Mg^{2+} as the activating ion.

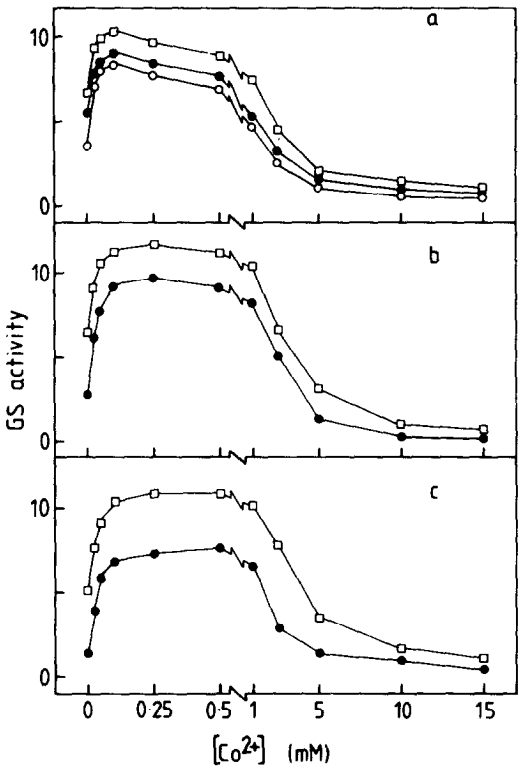

Fig. 2. Effects of Co^{2+}, Mg^{2+} and ATP on the biosynthetic activity of *Anabaena cylindrica* GS. Assays were performed in the presence of 1 mM ATP (a), 2.5 mM ATP (b), 5 mM ATP (c); 2.5 mM Mg^{2+} (○), 5 mM Mg^{2+} (●) and 10 mM Mg^{2+} (□). GS activity is expressed as μmol.P_i released.min^{-1}.(mg protein)$^{-1}$.

The effect of Co^{2+} on the apparent Michaelis constant of GS for NH_4^+: We examined the effect of Co^{2+} on K_m^{app} for NH_4^+ of GS since enzymic efficiency *in vivo* depends both on enzyme activity *per se* and on the ability of the enzyme to scavenge NH_4^+. As Fig. 3 shows, the K_m for NH_4^+ at 25 mM Mg^{2+} alone was 0.8 mM, a value unaffected by added Co^{2+}. At 5 mM Mg^{2+} alone, the K_m for NH_4^+ was 0.05 mM and at 5 mM Mg^{2+} + 0.1 mM Co^{2+} it was 0.1 mM. That is, Co^{2+}, at concentrations which activated the enzyme (see Fig. 1), had little effect on the low K_m for NH_4^+ obtained at low Mg^{2+} concentrations. The K_m^{app} values for ATP (0.17 ± 0.04 mM) and glutamate (2.5 ± 0.88 mM) were not significantly altered by such treatments. Thus although low (physiological, see ref. 13) Mg^+ concentrations may result in reduced GS activity, this can be counter-balanced physiologically by a decreased K_m for NH_4^+. However, on

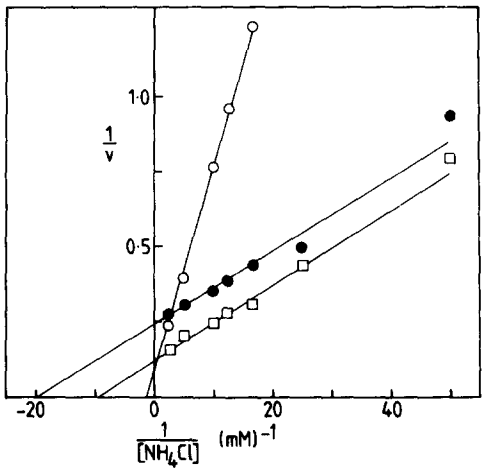

Fig. 3. Double reciprocal plot of the effect of NH_4Cl concentration on the biosynthetic activity of GS from *Anabaena cylindrica*. Assays were performed in the presence of 25 mM Mg^{2+} (○), 5 mM Mg^{2+} (●) and 5 mM Mg^{2+} plus 0.1 mM Co^{2+} (□).

adding Co^{2+} at low Mg^{2+} concentrations, high GS activity coupled with a low K_m for NH_4^+ results, thus increasing the NH_4^+-scavenging efficiency of the enzyme.

Inhibition of Mg^{2+}-dependent activity by Mn^{2+} and Ca^{2+}: Table 1 showed that Mg^{2+}-dependent activity was inhibited most strongly by Mn^{2+}, Hg^{2+} and Co^{2+} at the lowest concentrations tested (50 μM). Further tests were carried out on Mn^{2+}, a strong inhibitor of the GS of *Anabaena* and other organisms (see 6,11,14), and Ca^{2+} which plays an important role in enzyme regulation (see 15). Mn^{2+} caused a 50% inhibition of Mg^{2+}-dependent activity at 40 - 50 μM (Fig. 4a); this inhibitory effect was independent of Mg^{2+} concentrations up to 25 mM (the highest concentration tested). With Ca^{2+} (Fig. 4b) inhibition was relatively weak and was greatest at the lowest Mg^{2+} concentration tested suggesting a competitive inhibition of Mg^{2+}-dependent activity by Ca^{2+}. That is, Mn^{2+} and Ca^{2+} appear to have different modes of inhibition.

Stimulation of Mg^{2+}-dependent activity by adding the chelator EGTA: Having shown that Co^{2+} stimulated GS activity at low Mg^{2+} concentrations (Fig. 1a) whereas other cations tested inhibited GS (Fig. 4a) experiments were carried out in which the chelator EGTA was added to determine its effect on GS activity. GS activity (Fig. 1a) which was sub-optimum at low Mg^{2+} concentrations,

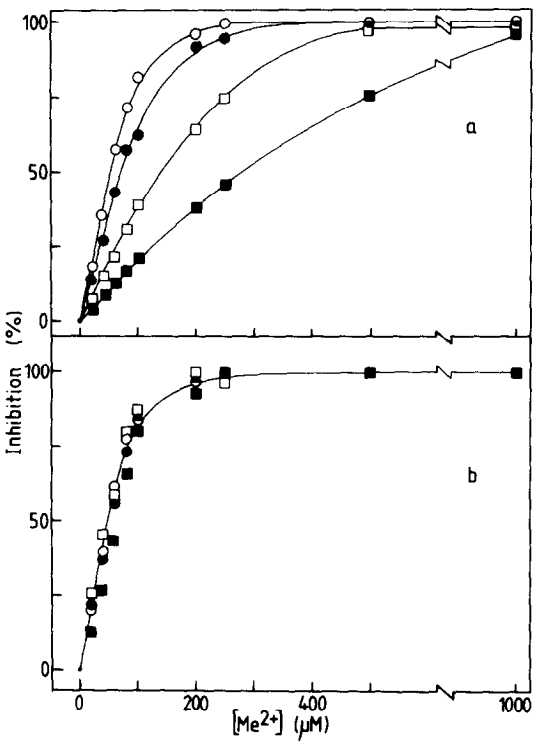

Fig. 4. Effects of Ca^{2+} (a) and Mn^{2+} (b) on *Anabaena cylindrica* GS activity in the presence of 2.5 mM (○), 5 mM (●), 10 mM (□) and 25 mM (■) Mg^{2+}. Control activities in the absence of Ca^{2+} or Mn^{2+} were 2.1, 5.2, 7.5 and 9.8 μmol.P_i released.min^{-1}.(mg protein)$^{-1}$ in the presence of 2.5 mM, 5 mM, 10 mM and 25 mM Mg^{2+}, respectively.

increased on EGTA treatment with only 1 mM Mg^{2+} being required for half maximum activity and with full activity occurring at 5 - 10 mM Mg^{2+}. EGTA thus mimics the effect of adding Co^{2+}. The data suggest that GS activity at low Mg^{2+} concentrations is inhibited by the presence of another cation, such as Mn^{2+} (see above), which can be removed by EGTA treatment or which is less inhibitory when Co^{2+} is present; possibly this inhibitory cation and Co^{2+} compete for a similar site (see also 6). Overall our data suggest several things. First, cyanobacterial GS may possess more than one type of cation-binding site - a catalytic site involved in substrate binding and a regulatory site (s) to which activating and inhibitory cations bind. At physiological pH (7.0 - 8.5) Mg^{2+} alone supports greatest GS activity and may be the natural substrate. The high Mg^{2+}:ATP ratio required for maximum activity suggests that Mg^{2+} plays a role other than in ATP binding. Variations in Mg^{2+}

availability, even within the physiological concentration range (see 13), result in large changes in GS activity, suggesting a regulatory function.

Second, Mn^{2+} may also have a regulatory function because although supporting little GS activity alone and only at non-physiological pH, it also inhibits Mg^{2+}-dependent activity. There is a recent report (7) that ovine brain GS exists as a Mn^{2+} enzyme; the essentiality of Mn^{2+} for the cyanobacterial enzyme awaits elucidation.

Third, a stimulatory effect of Co^{2+} on N_2-fixation has been noted (see 16,17) and is attributed to an effect on vitamin B_{12} metabolism. Vitamin B_{12} (cyanocobalamin) (0.01 - 100 μM) had no effect on GS activity. Possibly the observed effect of Co^{2+} on N_2-fixation is due in part to a stimulation of GS activity by free Co^{2+}. By increasing GS activity it could enhance NH_4^+ scavenging and relieve the inhibitory and repressive effects of NH_4^+ on nitrogenase (see 1). Relatively high Co^{2+} was required (0.1 mM) for stimulation thus necessitating a high intracellular concentration of this rare metal if it were to be so involved *in vivo*. It is also thought unlikely that, in general, Co^{2+} plays a regulatory role by binding directly to proteins (15) and it may be that Co^{2+} simply functions indirectly as an activator of GS by replacing inhibitory cations. The stimulation of Mg^{2+}-dependent activity of both purified GS (Fig. 1) and cell-free extracts (our unpublished data) by the chelator EGTA supports this possibility.

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