Purification, localization, properties and regulation of glutamine synthetase from *Hyphomicrobium X*

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The purification, location, properties and regulation of glutamine synthetase (GS) from the facultative methylotroph *Hyphomicrobium X* were investigated. The enzyme was purified to homogeneity by differential centrifugation, Blue Sepharose CL-6B chromatography and Sephadex G-25 gel filtration. The specific activity of the purified enzyme was $44.7 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$. GS was cytoplasmic in location with no direct association with DNA, membranes or the enzyme glutamine:2-oxoglutarate aminotransferase (GOGAT). The molecular mass of the native enzyme was 638 kDa; it was composed of 12 subunits of molecular mass 53 kDa. Double reciprocal plots showed that the enzyme followed Michaelis–Menten kinetics. The apparent K_m values for hydroxamate and glutamine in the γ -glutamyl transferase assay were 7.0 and 5.0 mM respectively; those of ammonia, glutamate and ATP in the biosynthetic assay were 0.032, 31.1 and 0.37 mM respectively. GS activity was controlled by covalent modification, the presence of specific divalent cations and feedback inhibition by several end-products of glutamine metabolism.

Introduction

Hyphomicrobium X is a facultative methylotroph which utilizes ammonium nitrogen as the preferred nitrogen source but can use nitrate and organic nitrogen, in particular, methylamine (Harder & Attwood, 1978; Brooke *et al.*, 1987). Irrespective of the primary source, nitrogen is assimilated into biomass via the intermediates glutamine and glutamate. There are two major pathways whereby nitrogen assimilation occurs in bacteria: one pathway functions under conditions of excess ammonium by utilizing the activity of an NADPH-dependent glutamate dehydrogenase and the other under conditions of ammonium limitation and involves the combined action of two enzymes, glutamine synthetase (GS) and glutamate:2-oxoglutarate aminotransferase (GOGAT). Both of these pathways have been reviewed extensively (Tyler, 1978; Dalton, 1979; Brown, 1980) and shown to be expressed in *Hyphomicrobium X* (Brooke *et al.*, 1987).

The most detailed studies on GS/GOGAT have focused on GS activity in *Escherichia coli* (Ginsburg & Stadtman, 1973; Tempest *et al.*, 1973). The ability of GS

to form a coupling reaction with GOGAT, together with its low K_m for ammonia, enables ammonium nitrogen to be assimilated when low concentrations are present in the environment. Moreover, GS responds to changes in the concentration of ammonia available. The activity levels are regulated by a complex control system which results in extremely rapid and precise responses to changes in the nitrogen status within the cell. When the ammonia concentration is high, GS loses its ability to form glutamine in the biosynthetic reaction

although the enzyme retains activity in the artificial assay

This rapid decrease in ability to form glutamine in reaction (1) is due to the attachment of adenylyl groups to the enzyme. This addition of up to 12 molar equivalents of AMP to the enzyme is reversible and due to the covalent attachment of AMP to a specific tyrosyl residue on each subunit in the enzyme structure (Kingdom *et al.*, 1967; Wulff *et al.*, 1967). This adenylation process is now known to be controlled via a complex cascade

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Abbreviations: GS, glutamine synthetase; GOGAT, glutamate:2-oxoglutarate aminotransferase.

system involving the enzymes adenylyltransferase, uridyltransferase and a regulatory protein (PII) which occurs in two forms, PIIA and PIID (Stadtman *et al.*, 1970; Shapiro & Stadtman, 1970; Ginsburg & Stadtman, 1973). Indeed, the adenylylation state of the enzyme can be altered to a specific level *in vitro* by incubation of GS with adenylyltransferase, PII, and various effectors including ATP, UTP, P_i , 2-oxoglutarate, glutamine and Mg^{2+} or Mn^{2+} . The final adenylylation state obtained is dependent not only on the ratio of PIIA:PIID, but also on which effectors are present and their concentration (Segal *et al.*, 1974). The adenylylation state has a considerable effect on the enzyme activity, resulting in changes in the K_m and V_{max} values as well as the pH optimum, divalent cation specificity and inhibitory properties (Ginsburg, 1969; Ginsburg *et al.*, 1970). The presence of certain divalent cations is essential for catalytic activity. In the absence of any divalent cation the GS is 'relaxed' and the structure less compact, the -SH groups are exposed and the enzyme is more susceptible to disaggregation (Shapiro & Stadtman, 1967). When GS in this form is incubated with divalent cations the -SH groups become buried in the enzyme structure and the catalytic activity is restored (Kingdom *et al.*, 1968; Valentine *et al.*, 1968; Segal & Stadtman, 1972b). It has been demonstrated, using the γ -glutamyltransferase assay (reaction 2), that GS is specific in the metal ion requirement for activity: Mn^{2+} supports the activity of both adenylylated (biosynthetically inactive) and deadenylylated (biosynthetically active) enzyme although the pH optimum varies considerably with the state of adenylylation. Addition of Mg^{2+} to the Mn^{2+} -dependent transferase assay inhibits the activity of the adenylylated subunits only (Shapiro & Stadtman, 1970; Stadtman *et al.*, 1970). This is used to measure the relative adenylylation state of the enzyme by comparing the activity in the presence of Mn^{2+} with the activity in the presence of Mn^{2+} and Mg^{2+} .

A second method of GS regulation involves cumulative feedback inhibition by the endproducts of glutamine metabolism (Woolfolk *et al.*, 1964; Hubbard & Stadtman, 1967a; Kleinschmidt & Kleiner, 1978). This appears to be the only regulatory mechanism to control GS activity in those organisms for which adenylylation control has not been demonstrated (Deuel & Stadtman, 1970; Wedler *et al.*, 1976).

Although the presence of GS has been reported in methanotrophs (Shishkina & Trotsenko, 1979) and in methylotrophic bacteria (Loginova *et al.*, 1982; Bellion & Bolbot, 1983; Brooke *et al.*, 1987), very little is known about the properties and regulation of this enzyme in methylotrophic bacteria. This paper describes the purification, location and regulation of GS from *Hypomicrobium X*.

Methods

Organism and growth conditions. *Hypomicrobium X* was maintained and grown in batch culture as described previously (Harder *et al.*, 1973). In order to ensure (i) expression of GS, the growth medium contained methanol (40 mM) as the carbon source and KNO_3 (10 mM) as the nitrogen source; (ii) that GS was predominantly in the adenylylated form (70% adenylylated), the standard growth medium was used to which $(NH_4)_2SO_4$ (100 mM) was added 1 min before harvesting the cells; (iii) that GS was predominantly in the deadenylylated form (13% adenylylated), the nitrate concentration in the medium was lowered to 0.5 mM. All media were inoculated with cells grown to the mid-exponential phase of growth in the same medium. Growth was followed by measuring the optical density at 430 nm; bacteria were harvested in the mid-exponential phase of growth, washed once with 50 mM-Tris/HCl buffer, pH 7.5 and used immediately.

Preparation of cell-free extracts. Cells were suspended (25%, w/v) in cold 50 mM-Tris/HCl buffer, pH 7.5. Ice-cold cell suspensions were disrupted by sonication for 10×30 s (6 μ m peak) alternating with 30 s cooling periods. Cell debris was removed by centrifugation (30000 g; 30 min at 4 °C) and the supernatant obtained was used as the crude cell-free extract.

Enzyme assays. All enzyme activities were measured at 30 °C. The observed enzyme rates were proportional to the amount of extract added and were linear for at least 3 min. The enzyme activities are given as μ mol product min^{-1} (mg protein) $^{-1}$.

GS [L-glutamate: ammonia ligase (ADP-forming); EC 6.3.1.2] was measured using either the γ -glutamyltransferase assay or the biosynthetic assay. (i) GS γ -glutamyltransferase activity was measured by the method of Bender *et al.* (1977), except that the pH was adjusted to 6.7 and the incubation was at 30 °C. (ii) The GS biosynthetic assay was a discontinuous assay which measured the release of P_i from ATP. The assay system was a modification of that described by Murrell & Dalton (1983). The pH of the buffer was increased to 7.5 and the concentration of ATP increased to 5 μ mol ml^{-1} . Samples (200 μ l) were removed from the reaction mixtures at appropriate time intervals and the P_i released was measured by the method of Baginski *et al.* (1967). The amount of P_i released from ATP was determined from a standard curve prepared by using known concentrations of P_i and following the same analysis procedure. Published assays were used to assay hydroxypyruvate reductase (EC 1.1.1.81) by the method of Blackmore & Quayle (1970), and NAD^+ -dependent formate dehydrogenase (EC 1.2.1.2) by the method of Johnson & Quayle (1964) as modified by Harder *et al.* (1973). Succinate dehydrogenase (EC 1.35.99.1), the NADH oxidase system and GOGAT (EC 1.4.1.14) were assayed as described by Meers *et al.* (1970).

Protein estimations. Protein concentrations in cell-free extracts were measured using the Lowry method with bovine serum albumin as standard.

Sucrose gradient centrifugation. Sucrose gradients (20–60%, w/v) were prepared in 50 mM-Tris/HCl buffer containing mercaptoethanol (2 mM) and $MnCl_2$ (1 mM) by successively layering less dense sucrose solution (2 ml) in 10% increments. Crude cell-free extracts (1.4 ml) were layered onto the top of the gradient and centrifuged in a Beckman L8 ultracentrifuge with a swinging bucket SW41 Ti rotor. After centrifugation the gradients were fractionated (from the top) into aliquots (0.5 ml). The sucrose concentration was determined using a refractometer. A calibration curve was constructed from sucrose solutions of known concentrations.

Enzyme purification.

All procedures were carried out at 4 °C. The buffer used throughout was 50 mM-Tris/HCl, pH 7.5, containing mercaptoethanol (2 mM),

MnCl₂ (1 mM) and EDTA (0.5 mM). Differential sedimentation was carried out using a Beckman L8 ultracentrifuge with a fixed-angle type 65 rotor into which rigid screw cap polycarbonate tubes (10 ml) were placed.

Step 1. Crude cell-free extracts (4 ml) were centrifuged at 100 000 *g* for 50 min and the supernatant (S1) was retained.

Step 2. The S1 fraction was centrifuged at 107 000 *g* for 140 min, and the supernatant (S2) was discarded. The pellet (P2) was gently resuspended in the buffer and allowed to solubilize for 18 h at 4 °C. Particulate material was removed by a slow speed centrifugation (35 000 *g*, 10 min) and the supernatant (RP2) retained.

Step 3. The RP2 fraction was subjected to a repeat of step 2 to give a second resuspended pellet (RP22).

Step 4. RP22 was loaded onto a column of Blue Sepharose CL-6B (1.0 × 2.0 cm) prepared for use according to the manufacturer's instructions, packed at a flow rate of 40 ml h⁻¹ and equilibrated with buffer. The column was washed at a flow rate of 30 ml h⁻¹ with buffer containing NaCl (1 M) to remove any weakly bound protein, then eluted by buffer containing ATP (20 mM). Fractions which contained GS activity were pooled.

Step 5. The pooled active fractions were separated from contaminating ATP by loading onto a column of Sephadex G-25 (2 × 6 cm). The column was equilibrated with buffer and eluted at a flow rate of 120 ml h⁻¹. Fractions containing activity were pooled and, if necessary, concentrated by forced dialysis against dry carboxymethylcellulose to a protein concentration of 0.1–0.5 mg ml⁻¹. Enzyme preparations from step 3 and step 5 could be stored at –20 °C for up to 6 months and the activity retained was 90 ± 5% of the original activity.

Electrophoretic methods.

All gels consisted of a stacking gel on top of a separating gel which were prepared and run using a discontinuous buffer system (Laemmli, 1970). Electrophoresis was at a constant current of 20 mA through the stacking gel and 40 mA through the separating gel. The apparatus was run at room temperature with denaturing SDS-gels and at 4 °C for the non-denaturing gels. A heat exchanger was used in the lower buffer chamber in order to minimize the increase in temperature during the separation. Denaturing SDS-gels were used to determine the purity of the enzyme preparation and size of enzyme subunits. Non-denaturing gels were used to estimate the molecular mass of the native enzyme and for the activity stain.

Denaturing (SDS-PAGE). This was done as described by Laemmli & Favre (1973), with the following calibration protein reference markers: bovine serum albumin (molecular mass 66 kDa), chicken egg albumin (45 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, subunit (36 kDa), bovine erythrocyte carbonic acid anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa) and bovine milk α -lactalbumin (14.2 kDa).

Non-denaturing gels (minus SDS). These contained, in the separating gels, either 3.0 or 5.5% (w/v) acrylamide, and in the stacking gel, 1.4% (w/v) acrylamide. Samples were prepared by the addition of an equal volume of 50 mM-Tris/HCl buffer, pH 6.7, containing equal quantities of glycerol and bromophenol blue (0.025%, w/v). The electrode buffer contained Tris (0.6 g l⁻¹) and glycine (2.88 g l⁻¹) at pH 8.3. The calibration protein reference markers used were: horse spleen ferritin (trimer molecular mass 1428.9 kDa, dimer 952.6 kDa and monomer 476.3 kDa); horse spleen apoferritin (dimer 886 kDa and monomer 443 kDa), Jack bean urease (tetramer 480 kDa and dimer 240 kDa), bovine serum albumin (dimer 132 kDa and monomer 66 kDa), chicken egg albumin (45 kDa) and bovine erythrocyte carbonic acid anhydrase (29 kDa). All gels, except those for demonstration of activity, were stained with Coomassie Brilliant Blue.

Activity stain. GS activity was detected using the γ -glutamyltransferase reaction mixture previously described except that the pH was increased to 7.5. Gels were removed from the PAGE apparatus and

rinsed in ice-cold 0.1 M-Tris/HCl buffer, pH 7.5, before incubation in the reaction mixture for 45 min at 30 °C. The gel was incubated further in ferric chloride reagent for up to 15 min to allow the bands to develop. The activity bands were stable for only 5–10 min, so *R_F* values were measured immediately.

Control of covalent modification. Crude cell-free extracts of GS in the deadenylylated form were prepared and incubated at 30 °C in the presence and absence of glutamine (5 mM), P_i (20 mM), Mn²⁺ (20 mM) and ATP (1 mM). These incubation mixtures were sampled at known time intervals and the adenylylation state measured with the γ -glutamyltransferase assay.

Inhibitor studies. The purified enzyme was preincubated in the presence of various products of glutamine metabolism (1.5 and 10 mM) in the standard biosynthetic assay mixture minus ATP. The adenylylated enzyme was assayed in the presence of Mn²⁺ and the deadenylylated enzyme in the presence of Mg²⁺ at their respective pH optima. Enzyme activity was measured after addition of ATP to the reaction mixture. Enzyme activities were compared with the control activity of the enzyme in the absence of any inhibitor.

Results and Discussion

Purification of the enzyme

The results of a typical purification are shown in Table 1 and Fig. 1. The enzyme was purified 91.2 fold with a 31.4% yield. The specific activity of the purified enzyme was 44.7 μ mol min⁻¹ (mg protein)⁻¹.

Purity of the enzyme

With non-denaturing PAGE a single band was developed which showed the enzyme had been purified to homogeneity. When the gels were stained for GS activity a single band was observed with an *R_F* value identical to that of the pure enzyme. (Data not shown.)

Location of GS within the cell

The crude cell-free extract was fractionated by sedimentation through a sucrose gradient (20–60%, w/v). When the fractions were monitored for absorbance at 260 and 280 nm, three peaks were revealed (Fig. 2a). Measurements for GS and GOGAT activity showed that the main activities of the two enzymes were recovered from the gradient in different fractions and that these activities were not associated with the fractions containing membrane-associated enzymes (Fig. 2b). Therefore the rapid differential sedimentation of GS activity in the purification procedure was unlikely to be due to complex formation with GOGAT (Streicher & Tyler, 1980) or an association with the cell membrane (Kleinschmidt &

Fig. 1. SDS-polyacrylamide gel of the purification stages of GS. A 10% (w/v) SDS-polyacrylamide gel was prepared and protein samples electrophoresed as described in Methods. Protein was visualized by staining with Coomassie Brilliant Blue. Protein markers (molecular mass values indicated) were as described in Methods. Tracks (3) and (9), protein markers; (2) and (8), crude cell-free extract (100 μ g); (7) S1 (100 μ g); (6) S2 (100 μ g); (5) RP22 (35 μ g); (4) Blue Sepharose fraction (4 μ g); (1) Blue Sepharose fraction (8 μ g).

Table 1. *Purification of GS from Hyphomicrobium X*

All purification steps were done as described in Methods. Enzyme activity was determined using the γ -glutamyltransferase assay.

Purification stage	Vol. (ml)	Total protein (mg)	Total activity (μ mol min ⁻¹)	Specific activity [μ mol min ⁻¹ (mg protein) ⁻¹]	Purification (-fold)	Yield (%)
Crude extract	8	105.6	51.2	0.49	1	100
RP22 fraction	5	17	50.65	3.00	6.1	98.9
Blue Sepharose chromatography	2	0.37	10.04	27.3	55.71	19.6
Sephadex G-25 filtration	3.2	0.36	16.06	44.7	91.2	31.4

Kleiner, 1978; Florencio & Romos, 1985). When the experiment was repeated with a crude cell-free extract which had been preincubated with DNAase I (150 μ g ml⁻¹) at 37 °C for 10–15 min the total absorbance at

260 nm recovered from the sucrose gradient was reduced significantly but the activity peaks were in identical positions, the GS activity sedimented equally well and the final enzyme purification and yield was comparable

Fig. 2. Ultracentrifugation of crude cell-free extracts on a 20–60% (w/v) sucrose gradient. Crude cell-free extract from *Hyphomicrobium X* grown in batch culture on methanol (40 mM) and KNO_3 (10 mM) was subjected to ultracentrifugation as described in Methods. (a) Absorbance at 280 nm (○) and at 260 nm (●); □, sucrose concentration. (b) Enzyme activities [$\mu\text{mol product min}^{-1} (\text{mg protein})^{-1}$; see Methods] in the fractions shown in (a). ○, Hydroxypyruvate reductase; △, formate dehydrogenase; ■, GOGAT; △, GS; ●, NADH oxidase; □, succinate dehydrogenase.

to that obtained without DNAase treatment (data not shown). Thus in *Hyphomicrobium X* the enzyme is cytoplasmic and the rapid differential sedimentation cannot be explained by an association with DNA or membranes.

Molecular mass

The molecular mass of the purified native enzyme was estimated using non-denaturing PAGE to be 638 kDa. This value is similar to values cited for GS from other bacteria (Table 2). When SDS-denaturing gels were used, two protein bands were observed with molecular masses of 52.5 and 53.7 kDa. Two protein bands of similar molecular mass have been reported with GS from a number of different bacteria (*Pseudomonas putida*, Streicher & Tyler, 1980; *Caulobacter crescentus*, Ely *et al.*, 1978; *Escherichia coli* and *Klebsiella aerogenes*, Bender & Streicher, 1979). Initially, the presence of the bands was thought to reflect a slight difference in the electrophoretic properties of the adenylylated and deadenylylated subunits. However, not all bacteria which possess a GS subject to covalent modification show subunits of different electrophoretic mobilities (*Azotobacter vinelan-*

dii, Kleinschmidt & Kleiner, 1978; *Nitrobacter agilis*, Kumar & Nicholas, 1984; *Rhodomicrobium vannielii*, Caballero *et al.*, 1987; *Vibrio alginolyticus*, Bodusing *et al.*, 1985). This suggests that the two bands are not a reflection of covalent modification of the GS subunits. When an average molecular mass was taken from the two protein bands (53.1 kDa) and compared with the molecular mass of the native enzyme (638 kDa), the enzyme was shown to be an oligomeric enzyme with twelve subunits (Table 2). Thus the size and structure of the GS purified from *Hyphomicrobium X* was consistent with that reported for the majority of the GS enzymes purified from prokaryotic sources.

Kinetic properties

The purified deadenylylated enzyme was used to measure the enzyme activity in the γ -glutamyltransferase and biosynthetic assays over a range of substrate concentrations. Double reciprocal plots showed that the enzyme followed Michaelis–Menten kinetics. The apparent K_m values for hydroxylamine (7.0 mM) and glutamine (5.0 mM) were similar to those reported for GS from the methanotroph *Methylococcus capsulatus* strain

Table 2. Comparison of the properties of GS from different micro-organisms

Enzyme source	Mol. mass (kDa)		No. of subunits	Covalent modification	Apparent K_m values (mM)						Reference*
	Native	Subunit			Biosynthetic (Mg ²⁺) assay		γ -Glutamyltransferase assay				
					NH ₄ ⁺	Glutamate	ATP	NH ₂ OH	Glutamine	ADP	
<i>Escherichia coli</i>	600	50	12	+	1.8	2.40	0.68	4.0	3.6	—	(1)
<i>Nitrobacter agilis</i>	700	58	12	+	0.2	6.30	—	2.6	11.6	—	(2)
<i>Bacillus stearothermophilus</i>	612	51	12	+	1.0	1.5	0.30	0.50	—	—	(3)
<i>Bacillus subtilis</i>	600	50	12	+	0.4	0.8	0.20	2.3	7.8	0.03	(4)
<i>Azotobacter vinelandii</i>	640	53	12†	+	0.15	1.20	0.94	—	—	—	(5)
<i>Anabaena</i> 7120	610	51	12	—	<0.02	2.10	0.32	—	—	—	(6)
<i>Methanobacterium ivanovi</i>	600	50	12	—	—	—	—	6.8	22.7	0.35	(7)
<i>Methylococcus capsulatus</i>	617	60	10	+	0.72	2.25	0.60	7.0	5.0	0.15	(8)
<i>Hyphomicrobium</i> X	638	53	12	+	0.032	3.10	0.37	7.0	5.0	—	(9)

* (1) Woolfolk *et al.* (1966); (2) Kumar & Nicholas (1984); (3) Wedler & Hoffman (1974); (4) Deuel & Stadtman (1970); (5) Kleinschmidt & Kleiner (1978); (6) Orr & Haselkorn (1981); (7) Bhatnagar *et al.* (1986); (8) Murrell & Dalton (1983); (9) present study.

† Active groups of 8, 10 and 24 subunits are present to a lesser extent.

Table 3. *Effect of certain metabolites on the adenylylation state of GS in crude cell-free extracts*

Cell-free extracts of *Hyphomicrobium X* grown in batch culture on methanol (20 mM) and $(\text{NH}_4)_2\text{SO}_4$ (1 mM) were incubated in the presence or absence of glutamine (5 mM), P_i (20 mM), Mn^{2+} (20 mM) and ATP (1 mM). Extracts were sampled and assayed for their adenylylation state.

Time (min)	Adenylylation state (%)	
	Incubation in presence of metabolites	Incubation in absence of metabolites
0	4.3	4.3
10	20	4.6
20	26.8	7.9
30	30	5
40	33	5
60	34	5.7
70	34.3	5.4

Bath (Murrell & Dalton, 1983). An apparent K_m for ADP could not be determined due to a high endogenous rate. At present there is no explanation for this high endogenous rate. The kinetic data for the biosynthetic assay (see Table 2) showed that the K_m value for ammonia (32 μM) was significantly lower than that for either glutamate (3.1 mM) or ATP (0.37 mM). This is in agreement with the physiological role of GS in the assimilation of ammonia under nitrogen-limited conditions. This value was lower than many reported values (0.1–1.0 mM) but not exceptional, since the apparent K_m for ammonia of GS from the cyanobacterium *Anabaena* 7120 was 20 μM (Orr & Haselkorn, 1981).

Regulation of GS

We have reported previously (Duchars & Attwood, 1989) that in *Hyphomicrobium X* GS synthesis is repressed when nitrogen is available to the cell in a plentiful and readily utilizable form and induced when nitrogen is limiting for growth. Moreover, GS activity is controlled by the system of covalent modification involving adenylylation and deadenylylation (Brooke *et al.*, 1987). This control mechanism itself is regulated in many bacteria via a complex cascade system that is stimulated and inhibited by specific metabolites (Brown *et al.*, 1971; Mangum *et al.*, 1973). Crude cell-free extracts containing GS in the deadenylylated state were incubated at 30 °C in the presence and absence of glutamine, P_i , Mn^{2+} and ATP and the adenylylation state of the enzyme was measured. The adenylylation state of the enzyme increased from 4 to 34% over a 70 min incubation period (Table 3) in the presence of metabolites, compared to no change in the adenylylation state when incubated under the same conditions without added metabolites. This

Table 4. *Effect of addition of divalent cations to the standard γ -glutamyltransferase assay*

Purified adenylylated and deadenylylated enzyme were incubated in the standard γ -glutamyltransferase assay mixture in the presence and absence of extra divalent cations. Activity of the adenylylated enzyme in the standard assay mixture alone was 2.86 $\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$ and of the deadenylylated enzyme 3.22 $\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$.

Divalent cation	Concn (mM) ...	Inhibition (%)					
		Adenylylated enzyme			Deadenylylated enzyme		
		3	6	10	3	6	10
Mn^{2+}		1	1	4	3	6	5
Mg^{2+}		18	30	38	4	3	8
Cu^{2+}		17	29	73	30	50	85
Co^{2+}		25	42	75	17	39	76
Zn^{2+}		25	50	80	22	35	82
Ca^{2+}		32	41	54	24	33	48
Fe^{2+}		35	70	83	25	55	92

suggests that the covalent modification of GS in *Hyphomicrobium X* can be regulated in a manner similar to that reported for *E. coli* (Segal & Stadtman, 1972*a, b*; Ginsburg & Stadtman, 1973).

Effect of divalent cations on GS activity

The divalent cation requirement for catalytic activity has been found to depend on the adenylylation state (Kingdom *et al.*, 1967). When the γ -glutamyltransferase assay was used to measure GS activity with the purified enzyme from *Hyphomicrobium X*, the results showed that, irrespective of the adenylylation state, none of the divalent cations tested (Co^{2+} , Ca^{2+} , Fe^{2+} or Zn^{2+}), except Mg^{2+} , could replace Mn^{2+} in the standard assay mixture. When Mg^{2+} was substituted for Mn^{2+} the activity was 50% of that measured in the presence of Mn^{2+} . Furthermore, when divalent cations were added at increasing concentrations (0–10 mM) to a standard assay mixture containing Mn^{2+} , all the divalent cations tested, except Mg^{2+} , inhibited to a similar extent both the adenylylated and the deadenylylated form of the enzyme (Table 4). Mg^{2+} inhibited the adenylylated form of the enzyme only. The effect of adding divalent cations to the more physiologically relevant biosynthetic assay was studied with the partially purified enzyme (RP22 stage), prepared from cells which contained GS predominantly in either the adenylylated or the deadenylylated state. When the activity of the deadenylylated form of the enzyme was measured over the pH range 6.5–8.5 with different divalent cations added, activity could be detected only in the presence of Mg^{2+} and the pH

Table 5. *Feedback inhibition of purified GS by products of glutamine metabolism*

Percentage inhibition was calculated with respect to control activity in the absence of any added inhibitor. Deadenlylated control activity was $6.38 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$; adenlylated control activity was $3.75 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$.

Metabolic product	Concn (mM) ...			Deadenlylated enzyme			Adenlylated enzyme		
	1	5	10	1	5	10	1	5	10
Glycine	9	43	62	0	0	0	0	0	0
Alanine	35	69	81	7	22	32	7	22	32
Serine	9	52	68	12	19	33	12	19	33
Tryptophan	0	0	7	0	5	5	0	5	5
Histidine	0	0	6	0	12	22	0	12	22
Glutamine	0	0	0	7	12	12	7	12	12
Carbamyl phosphate	42	59	75	42	65	82	42	65	82
Glucosamine 6-phosphate	0	6	21	0	14	28	0	14	28
AMP	0	22	33	17	49	79	17	49	79
CTP	0	21	96	0	74	80	0	74	80

Table 6. *Effect on deadenlylated GS of combinations of feedback inhibitors*

Percentage inhibition was calculated with respect to control activity $[6.38 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}]$ in the absence of any added inhibitor.

Inhibitor [Concn, mM]	Inhibition (%)	Inhibitor [Concn, mM]	Inhibition (%)	Inhibitor [Concn, mM]	Inhibition (%)	Calculated inhibition (%)		Observed inhibition with combination of inhibitors (%)
						Cumulative*	Antagonistic	
Ser [3]	45	Gly [3]	32	-	-	63	77	63
Ser [3]	41	Ala [3]	59	-	-	76	100	76
Ala [3]	60	Gly [3]	32	-	-	63	92	67
Ala [1]	35	Gly [1]	9	Ser [1]	9	46	53	46

* Calculated by using the formula $X + Y(100 - X)$, where X and Y represent the percentage inhibition caused by the modifiers x and y alone respectively (Woolfolk & Stadtman, 1967).

optimum was 7.5. When the experiment was repeated with the enzyme in the adenylylated form, activity could be detected only in the presence of Mn^{2+} and the pH optimum was 6.7. Neither the adenylylated nor deadenylylated enzyme showed activity at the pH optimum of the other form of the enzyme. Therefore, the activity of GS purified from *Hyphomicrobium X* was controlled by covalent modification and like similar GS from other bacteria the Mn^{2+} activated biosynthetic assay and the Mg^{2+} -activated biosynthetic assay were specific to the adenylylated and deadenylylated form, respectively. Moreover, the activity of the deadenylylated Mg^{2+} -activated form of the enzyme was 1.5-fold greater than that of the adenylylated Mn^{2+} -activated form when measured at their respective pH optima. This effect has been reported with GS enzymes purified from other micro-organisms.

Feedback inhibition

The possibility of feedback regulation of GS by various amino acids and other endproducts of glutamine metabolism was investigated using both purified adenylylated and purified deadenylylated enzyme preparations. Glycine, alanine and serine had a greater inhibitory effect on the deadenylylated enzyme whilst histidine, glutamine and AMP were more inhibitory towards the adenylylated enzyme (Table 5). Both enzyme forms were not inhibited by tryptophan and equally inhibited by carbamyl phosphate, glucosamine 6-phosphate and CTP. From the level of inhibition observed with each inhibitor alone theoretical calculations can be made to predict the percentage inhibition that would be observed when combinations of two or more inhibitors were used and the overall inhibition was additive, antagonistic or cumulative (Woolfolk & Stadtman, 1967). The results (Table 6) demonstrated that the inhibition observed correlated closely with that predicted for cumulative feedback inhibition of two or more inhibitors. This can be taken as presumptive evidence that there were separate binding sites on the enzyme for each inhibitor and that there were no interactions between the sites (Woolfolk & Stadtman, 1967; Shapiro & Stadtman, 1970). The GS from *Hyphomicrobium X* is subject to feedback inhibition similar to that reported with GS from Gram-positive and other Gram-negative bacteria (Hubbard & Stadtman, 1967a, b; Wedler *et al.*, 1976). The addition of single endproducts caused partial inhibition whilst the presence of two or more inhibitors had a cumulative effect (Woolfolk & Stadtman, 1967). Moreover, the adenylylation state of the enzyme affected its susceptibility to feedback inhibitors.

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