Purification, Properties and Regulation of Glutamine Synthetase from *Nitrobacter agilis*

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Glutamine synthetase (EC 6.3.1.2) was purified about 430-fold from the nitrifying bacterium *Nitrobacter agilis* by affinity chromatography on Blue Sepharose CL-6B and gel filtration on Sepharose 4B. The enzyme (apparent mol. wt 700 000), which consisted of 12 subunits, each of mol. wt 58 000, required a divalent cation for both biosynthetic and transferase activities. Regulation of glutamine synthetase both by a feedback inhibition involving amino acids and by an adenylation/deadenylation mechanism was studied. The enzyme was highly adenylylated (75–90%) in cell free extracts from cells grown on nitrite as the sole source of nitrogen. The adenylylated form of the enzyme could be deadenylylated by treatment with snake venom phosphodiesterase. An isoactivity pH of 7.4 was recorded for glutamine synthetase.

INTRODUCTION

Glutamine synthetase [EC 6.3.1.2; L-glutamate: ammonia ligase (ADP forming)] is one of the key enzymes for ammonia assimilation in a variety of bacteria and has been the subject of several reviews (Shapiro & Stadtman, 1970; Meister, 1974; Tyler, 1978). The enzyme from *Escherichia coli* is regulated by a complex set of mechanisms (Magasanik *et al.*, 1974) involving feedback inhibition, repression and adenylation/deadenylation (Woolfolk *et al.*, 1966; Ginsburg & Stadtman, 1973; Wohlhueter *et al.*, 1973). In recent years, the properties of glutamine synthetase have been studied in a variety of bacteria (Bender *et al.*, 1977; Siedel & Shelton, 1979; Alef *et al.*, 1981; Alef & Zumft, 1981; Bhandari & Nicholas, 1981; Khanna & Nicholas, 1983). In general, glutamine synthetase from Gram-negative bacteria is regulated by an adenylation/deadenylation mechanism (Ginsburg & Stadtman, 1973). The unadenylylated form (GS) and the adenylylated form (GS-AMP) of the enzyme differ in their regulatory properties (Bender *et al.*, 1977; Alef & Zumft, 1981). It is well known that the adenylation state of the enzyme depends on the nitrogen source in the growth medium. Thus, cells grown with NH_4^+ contain glutamine synthetase largely in an adenylylated form (Wohlhueter *et al.*, 1973). The chemolithotrophic nitrifying bacteria usually rely on a specific nitrogen source for their nitrogen and energy requirements; for example *Nitrobacter* and *Nitrosomonas* utilize NO_2^- and NH_4^+ respectively. Although glutamine synthetase from *Nitrosomonas europaea* has been purified and some of its properties studied (Bhandari & Nicholas, 1981), little is known about its role in *Nitrobacter agilis*. Aspects of the metabolism of nitrifying bacteria have been reviewed on several occasions (Aleem, 1970, 1977; Kelly, 1971; Nicholas, 1963, 1978; Suzuki, 1974; Wallace & Nicholas, 1969). We now report on the purification, properties and regulation of glutamine synthetase from *N. agilis*.

Abbreviations: CTAB, cetyltrimethylammonium bromide; SVP, snake venom phosphodiesterase.

METHODS

Bacteria and growth conditions. Batch cultures (40 l) of *Nitrobacter agilis* ATCC 14123 were grown at 28 °C for 5 d in an inorganic medium (Wallace *et al.*, 1969) and harvested by continuous flow centrifugation (Sorvall) at 4 °C as described previously (Bhandari & Nicholas, 1979; Kumar & Nicholas, 1981). Harvested cells, washed several times with cold 10 mM-Tris/HCl, 1 mM-MnCl₂ buffer (pH 7.2) were finally suspended in an appropriate volume of the same buffer.

Preparation of cell extracts. Washed cells in 10 mM-Tris/HCl, 1 mM-MnCl₂ buffer (pH 7.2) were disrupted with an ultrasonic probe (20 kHz) with 2 min bursts over a period of 30 min at 4 °C in an ice bath. Sonicated extracts were centrifuged at 30000 g for 20 min at 4 °C. The supernatant (S₃₀) was used for the purification of glutamine synthetase.

Purification of glutamine synthetase. The S₃₀ fraction was heat-treated at 50 °C for 15 min with constant stirring, then chilled in ice for 15 min and centrifuged at 30000 g for 15 min. The supernatant contained all the glutamine synthetase activity. Longer heat treatment or higher temperatures resulted in a loss of enzyme activity. The supernatant was loaded onto a Blue Sepharose CL-6B column (1.5 × 9 cm) equilibrated against 10 mM-Tris/HCl, 1 mM-MnCl₂ buffer (pH 7.2). The column was then washed with buffer (flow rate 50 ml h⁻¹) until the absorbance (A₂₈₀) was close to zero. Glutamine synthetase was eluted from the column with 2 mM-ADP in the same buffer. Active fractions were pooled, dialysed against buffer overnight, concentrated on an Amicon PM-10 membrane and loaded onto a Sepharose 4B column (2 × 70 cm) equilibrated against the buffer. The enzyme was eluted with the same buffer (flow rate 12 ml h⁻¹) and the active fractions pooled and concentrated as before.

Polyacrylamide gel electrophoresis. This was carried out in 5 and 7% (w/v) polyacrylamide tube gels. The stacking gel was 3% (w/v) polyacrylamide in 125 mM-Tris/HCl buffer (pH 6.8) and the running gel 5 or 7% (w/v) polyacrylamide in 375 mM-Tris/HCl buffer (pH 8.8). The electrode buffer was 12.5 mM-Tris, 96 mM-glycine (pH 8.4). Electrophoresis was carried out at 2 mA per gel at constant current. Gels were stained with Coomassie brilliant blue R250.

Determination of molecular weight. The molecular weight of the native enzyme was determined by gel filtration in a Sepharose 6B column (1.6 × 100 cm) equilibrated with 50 mM-Tris/HCl buffer (pH 7.5) according to the method of Andrews (1970). The column was calibrated with aldolase (*M_r* 158000), catalase (*M_r* 232000), ferritin (*M_r* 440000) and thyroglobulin (*M_r* 669000) as marker proteins. The determination of subunit molecular weight was done by discontinuous gel electrophoresis in the presence of 0.1% (w/v) SDS using Tris/glycine buffer (pH 8.3) according to the methods of Laemmli (1970) and Weber & Osborn (1975). The gels were calibrated with the following protein standards: phosphorylase *b* (*M_r* 94000), albumin (*M_r* 67000), ovalbumin (*M_r* 43000), carbonic anhydrase (*M_r* 30000) and trypsin inhibitor (*M_r* 20100).

Enzyme assays. Both the γ -glutamyl transferase and the biosynthetic activities of glutamine synthetase were determined by the methods of Shapiro & Stadtman (1970). The reaction mixture in a final volume of 1 ml contained (mM): imidazole/HCl (pH 7.2 or 7.4), 40; glutamine, 30; hydroxylamine hydrochloride (neutralized with 2 M-NaOH), 30; MnCl₂ · 4H₂O, 0.5; sodium arsenate, 20; ADP, 0.4; and an appropriate quantity of enzyme. For the *in vivo* assay in whole cells, the assay mixture also contained 20 μ g cetyltrimethylammonium bromide (CTAB) ml⁻¹. Control tubes without glutamine and hydroxylamine, respectively, were always included. For biosynthetic activity the assay mixture in a final volume of 0.2 ml contained (mM): imidazole/HCl, pH 7.0, 50; glutamate, 100; NH₄Cl, 50; ATP, 10; MgCl₂, 5 and an appropriate quantity of enzyme. Glutamate was omitted from control tubes and a correction was also made for non-enzymic production of P_i from ATP. All incubations were at 37 °C, usually for 15–30 min.

Protein and ammonia determinations. Protein was determined by the method of Bradford (1976) using bovine serum albumin as a standard. Ammonia was assayed by the method of Ballantine (1957) using NH₄Cl as a standard.

Biochemicals. AMP, ADP, ATP, imidazole, γ -glutamyl hydroxamate (γ GH), snake venom phosphodiesterase (SVP) and various amino acids were purchased from Sigma. Molecular weight standards were from Pharmacia. All other chemicals were the best grade available. Double glass-distilled water was used throughout.

Chromatographic material. Blue Sepharose CL-6B, Sepharose 4B and Sepharose 6B were purchased from Pharmacia and were prepared for use according to the instructions from the manufacturer.

RESULTS

Purification and properties of glutamine synthetase

Glutamine synthetase was purified about 430-fold by affinity chromatography on Blue Sepharose CL-6B and gel filtration on Sepharose 4B (Table 1). The purified enzyme had a specific activity of 220 μ mol γ -glutamyl hydroxamate produced min⁻¹ (mg protein)⁻¹ and moved as a single major band in polyacrylamide gel electrophoresis under both denaturing and

Table 1. *Purification of glutamine synthetase*

All purification steps (except heat treatment) were performed at 4 °C as described in Methods. Enzyme activity was determined by following the production of γ -glutamyl hydroxamate from L-glutamine and NH_2OH at pH 7.2. One enzyme unit is defined as $\mu\text{mol } \gamma$ -glutamyl hydroxamate produced min^{-1} and specific activity as number of units (mg protein^{-1}).

Purification step	Total protein (mg)	Total units	Specific activity	Purification (fold)	Recovery (%)
Crude extract (S_{30})	148.56	75.81	0.51	1.0	100
Heat treatment	100.81	69.92	0.69	1.4	94
Pooled Blue Sepharose CL-6B fractions	2.10	45.33	21.59	42.3	60
Pooled Sepharose 4B fractions	0.20	44.0	220.0	431.0	58

Table 2. *Effect of NH_4^+ on K_m of the substrates of γ -glutamyl transferase activity of purified glutamine synthetase*

Transferase activity of the enzyme was measured as described in Methods except that the amounts of glutamine and NH_2OH were varied between 0 and 50 mM and NH_4Cl was included in the assay mixture as indicated. K_m values were determined from double reciprocal plots of rates of reaction against the initial substrate concentration according to Lineweaver and Burk. The data were analysed by a computer program (Cleland, 1967); SEM values are shown.

NH_4^+ concn (mM)	K_m (mM)	
	Glutamine	NH_2OH
0	11.6 ± 1.5	2.6 ± 0.8
10	18.2 ± 1.6	4.0 ± 0.3
20	23.8 ± 2.1	5.1 ± 0.1
30	27.8 ± 1.0	5.6 ± 0.1

non-denaturing conditions. The purified enzyme was stable for several weeks at -10°C . The approximate molecular weight of the enzyme was 700 000, while the subunit molecular weight determined in SDS gels was 58 000, indicating that the enzyme is composed of 12 homologous subunits. The K_m values for the substrates of the glutamyl transferase reaction, glutamine and NH_2OH , were $11.6 \text{ mM} \pm 1.5$ (SEM) and $2.6 \text{ mM} \pm 0.8$, respectively; and for the substrates of biosynthetic reaction, glutamate and NH_4^+ , were 6.3 ± 1.6 and 0.2 mM respectively. Ammonia competitively inhibited the transferase reaction and it decreased the affinity of the enzyme for both glutamine and NH_2OH (Table 2). Both the transferase and the biosynthetic activities required a divalent cation. Maximum transferase activity was recorded with Mn^{2+} (0.3–10 mM) and the order of effectiveness of the divalent cations (at 10 mM) was $\text{Mn}^{2+} > \text{Cu}^{2+} > \text{Mg}^{2+} > \text{Co}^{2+}$; for biosynthetic activity it was $\text{Mg}^{2+} > \text{Mn}^{2+} > \text{Zn}^{2+} > \text{Cu}^{2+} > \text{Ni}^{2+}$.

Feedback inhibition

Feedback inhibition of glutamine synthetase by various amino acids and nucleotides has been reported in many bacteria, e.g. *Escherichia coli* (Kingdon & Stadtman, 1967), *Bacillus licheniformis* (Hubbard & Stadtman, 1967), *Klebsiella aerogenes* (Bender *et al.*, 1977), *Rhodospseudomonas capsulata* (Johansson & Gest, 1976), *Nitrosomonas europaea* (Bhandari & Nicholas, 1981), *Chlorobium vibrioforme* (Khanna & Nicholas, 1983) and *Methylococcus capsulatus* (Murrell & Dalton, 1983). The purified enzyme from *Nitrobacter agilis* was similarly inhibited. Thus at 10 mM final concentration, alanine, serine, glycine and tryptophan inhibited transferase activity by 65, 45, 40 and 33%, and biosynthetic activity by 60, 30, 35 and 5%, respectively. When the inhibition data were plotted as double reciprocal plots of the fractional inhibition against the concentration of amino acids, the values of the intercepts on the y axes were > 1 , indicating that these amino acids only partially inhibited the enzyme activity at saturating concentrations. The combined effects of various amino acids on the transferase activity are shown in Table 3. The inhibition due to various combinations of amino acids was cumulative rather than additive,

Table 3. *Feedback inhibition of purified glutamine synthetase by amino acids*

Transferase activity of the enzyme was determined as described in Methods except that the reaction mixture also contained 5 mM of the appropriate amino acid, as indicated. Values for cumulative inhibition were calculated according to the method of Stadtman *et al.* (1968).

Amino acid(s)	Percentage inhibition		
	Observed	Cumulative	Additive
Ala	40	—	—
Gly	27	—	—
Ser	36	—	—
Ala + Gly	52	56	67
Ala + Ser	62	62	76
Ser + Gly	52	53	63
Ala + Gly + Ser	69	72	103

Table 4. *Effects of CTAB treatment on transferase activity in cell suspensions and in cell-free extracts (S₂₅)*

Exponentially growing cultures (1 l) were harvested in 250 ml polycarbonate bottles in a Sorvall-GSA rotor at 10000 *g* for 20 min at 4 °C. As indicated, CTAB was added to cultures (2.5 µg ml⁻¹) before harvesting. Cells washed once with cold 10 mM-Tris/HCl, 1 mM-MnCl₂ buffer (pH 7.2) were finally suspended in a small volume (approx. 5 ml) of the buffer. For *in vitro* assay, cell suspensions were sonicated for 20 min and then centrifuged at 25000 *g* for 15 min. The supernatant (S₂₅) and cell suspension were used to determine the transferase activity at pH 7.4 with and without 60 mM-MgCl₂, as described in Methods. Transferase activity is defined as µmol γ-glutamyl hydroxamate produced (30 min)⁻¹ (mg protein)⁻¹.

Assay	CTAB treatment	Transferase activity		+ Mg ²⁺ / - Mg ²⁺ ratio
		- Mg ²⁺	+ Mg ²⁺	
<i>in vitro</i>	—	10.50	2.10	0.20
	+	9.30	1.50	0.16
<i>in vivo</i>	—	13.50	2.25	0.17
	+	12.50	3.0	0.23

indicating that each modifier is completely independent in its action and thus it is possible that separate binding sites on the enzyme are present for each of the inhibitors, as proposed for the *E. coli* enzyme (Stadtman *et al.*, 1968).

Adenylation/deadenylation

In enteric bacteria (Stadtman *et al.*, 1970; Bender *et al.*, 1977), photosynthetic bacteria (Johansson & Gest, 1977; Alef & Zumft, 1981; Michalski *et al.*, 1983) and rhizobia (Darrow & Knotts, 1977) the extent of transferase activity in the presence of 60 mM-MgCl₂ has been used as an indication of the degree of adenylation of glutamine synthetase. The fully adenylylated enzyme is inactive in the presence of Mg²⁺, whereas the deadenylylated enzyme is not affected. Since the adenylation state of the enzyme can change during harvesting of the bacteria (Bender *et al.*, 1977) CTAB was added to cultures to stabilize the adenylation state of the enzyme. The data in Table 4 indicate that the enzyme from normally grown *N. agilis* cells was severely inhibited by Mg²⁺. The CTAB treatment of *N. agilis* cells prior to harvest had little effect on the extent of Mg²⁺ inhibition of transferase activity. Similar results were observed when cells harvested with or without CTAB were assayed for *in vivo* transferase activity. Based on a 12 subunit enzyme and applying the Shapiro & Stadtman (1970) formula [$E_n = 12 - 12 (+Mg^{2+} / -Mg^{2+})$], an adenylation state of 9 can be calculated for the *N. agilis* enzyme, and it varied between 8 and 11 in 20 separately grown batches of cells.

The effects of SVP treatment on transferase activity of glutamine synthetase, with and without 60 mM-MgCl₂, are shown in Fig. 1. In these experiments, transferase assays were carried out

Fig. 1

Fig. 2

Fig. 1. Effects of SVP treatment on purified glutamine synthetase. The pH of a sample of purified enzyme was adjusted to 8.8 with 1 M-Tris and then divided into two equal volumes. One was treated with SVP ($50 \mu\text{g ml}^{-1}$) at 37°C and the other used as a control. At the times indicated, samples of untreated and SVP-treated enzyme were withdrawn and assayed for transferase activity with and without 60 mM-MgCl_2 at pH 7.4. Enzyme activity is expressed as A_{540} . The enzyme preparation was diluted initially so that $50 \mu\text{l}$ of enzyme produced enough γ -glutamyl hydroxamate in 15 min to give an absorbance of about 0.15 (without added Mg^{2+}). Untreated enzyme with (●) and without (○) Mg^{2+} ; treated enzyme with (■) and without (□) Mg^{2+} .

Fig. 2. Determination of the isoactivity pH of purified glutamine synthetase. SVP-treatment ($20 \mu\text{g ml}^{-1}$) of purified enzyme was as described in Fig. 1. The transferase activity of treated and untreated preparations was determined at various pH values as described in Methods. Untreated glutamine synthetase (○), SVP-treated for 20 min (●), 40 min (□) and 60 min (■), respectively.

at pH 7.4 in the presence of 0.3 mM-MnCl_2 . The Mg^{2+} inhibition of transferase activity of purified glutamine synthetase was completely reversed after a 30 min treatment with SVP.

The adenylylated and unadenylylated forms of glutamine synthetase have different pH optima for transferase activity; the adenylylated form has a lower pH optimum (Bender *et al.*, 1977). By treating glutamine synthetase from *N. agilis* with SVP for defined periods, it was possible to prepare the enzyme at various stages of adenylylation as shown in Fig. 2. The native enzyme (without SVP treatment) had a pH optimum of around 7.0 and the deadenylylated form (SVP-treated for 60 min) at 7.8. The isoactivity point of the two forms of the enzyme was about 7.4 and this explains why the transferase activity of the enzyme was not affected by SVP treatment when assayed in the absence of Mg^{2+} (Fig. 1).

The two forms of enzyme have been shown to be inhibited differentially by feedback inhibitors (Ginsburg, 1969; Bender *et al.*, 1977). Similar results were observed with the *N. agilis* enzyme (Table 5). The data show that the deadenylylated form was subjected preferentially to a feedback inhibition by alanine, glycine and serine and the adenylylated form by $5'$ -AMP (Table 5). As the extent of inhibition by these feedback inhibitors was dependent on the degree of adenylylation of the enzyme, which varied slightly from batch to batch ($E_n = 9$ to 11), it is not possible to give error limits for the results in Table 5. For various batches of cells the pattern of inhibition was similar to that shown in Table 5, even though the extent of inhibition varied between batches. The SEM within a single batch of cells was never more than 5%.

DISCUSSION

Glutamine synthetase and glutamate synthase are key enzymes for the assimilation of ammonia in many bacteria grown with low concentrations of ammonia (Tyler, 1978). Glutamate dehydrogenase is usually important when micro-organisms utilize relatively high concentrations of ammonia. As expected, *Nitrosomonas europaea* grown with NH_4^+ contains glutamate dehydrogenase as the major enzyme for ammonia assimilation (Hooper *et al.*, 1967) and has

Table 5. *Effects of some amino acids and 5'-AMP on adenylylated and deadenylylated forms of the purified glutamine synthetase*

The deadenylylated form of glutamine synthetase was prepared by SVP treatment as described in Fig. 1, except that the incubation was for 1 h. The adenylylation state of the enzyme was checked by determining the effect of Mg^{2+} on transferase activity at pH 7.4 as described in Table 4. All amino acids were at a final concentration of 10 mM and AMP at 20 mM. The activities of untreated (GS-AMP) and SVP-treated (GS) enzyme were 215 and 190 $\mu\text{mol } \gamma\text{-glutamyl hydroxamate produced min}^{-1} (\text{mg protein})^{-1}$, respectively.

Additions	Percentage inhibition of transferase activity	
	GS-AMP	GS
L-Alanine	70	85
L-Glycine	35	60
L-Serine	40	55
5'-AMP	65	36

relatively little glutamine synthetase activity (Bhandari & Nicholas, 1981). On the other hand, *N. agilis*, an NO_2^- oxidizing bacterium, has appreciable activities of glutamine synthetase and glutamate dehydrogenase and little glutamate synthase (Kumar & Nicholas, 1982).

In this paper it is shown that the enzyme from *N. agilis* has properties similar to those of the enzyme from other bacteria, viz. requirement for divalent cations, molecular weight, number of subunits and inhibition by amino acids and NH_4^+ . The inhibition by NH_4^+ of the transferase activity of glutamine synthetase supports a postulated model that glutamine reacts with the enzyme in such a way that its $-\text{NH}_2$ group occupies the NH_4^+ binding site, while the 'oxygen binding' site to which glutamate is normally bound is required for the attachment of the corresponding oxygen group of glutamine (Gass & Meister, 1970). The results also indicate that the inactivation of the NH_4^+ binding site by glutamine would preclude the binding of NH_2OH at this locus.

It is of interest that the *N. agilis* enzyme was highly adenylylated even when the cells were grown with nitrite but without any ammonia in the culture medium. CTAB treatment of the cultures prior to harvest had no substantial effect on the state of adenylylation of glutamine synthetase. Relatively low concentrations of CTAB ($2.5 \mu\text{g ml}^{-1}$) were used compared to those used for other bacteria (Bender *et al.*, 1977; Davies & Ormerod, 1982; Michalski *et al.*, 1983) because higher concentrations resulted in cell lysis. This lysis may be associated with the low cell density of exponentially-grown cultures of *N. agilis*.

Cells of *N. agilis* contained approximately 30 mM intracellular NH_4^+ , assuming that the intracellular water space is about $1.2 \mu\text{l (mg dry wt)}^{-1}$ (Kumar & Nicholas, 1983). This would explain why glutamine synthetase is highly adenylylated so that glutamate dehydrogenase is the main route of NH_4^+ assimilation (Kumar & Nicholas, 1982, 1984). Ammonia accumulation in cells is possible if nitrite reductase is under minimal regulatory control.

The native adenylylated form of glutamine synthetase could be deadenylylated by SVP. This indicates that the *N. agilis* enzyme is indeed regulated by an adenylylation/deadenylation mechanism. Another line of evidence to support this conclusion is that differentially adenylylated forms of the enzyme differ in their pH optima. The isoactivity pH of 7.4 lies between the value 7.15 for *Escherichia coli* (Stadtman *et al.*, 1970) and 7.55 for *Klebsiella aerogenes* (Bender *et al.*, 1977). The isoactivity point of the *N. agilis* enzyme was independent of the purification stage of the enzyme as was also found in *K. aerogenes* (Bender *et al.*, 1977).

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