Purification and Regulation of a Cloned *Thiobacillus ferrooxidans* Glutamine Synthetase

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Glutamine synthetase (GS) (EC 6.3.1.2) was purified from an *Escherichia coli glnA* deletion strain containing the *Thiobacillus ferrooxidans* GS structural gene. The apparent M_r of the cloned *T. ferrooxidans* GS subunit was approximately 60000. This indicates a particle M_r for the undissociated enzyme of 720000, assuming the enzyme is the typical dodecamer. Electron microscopy of purified GS revealed characteristic disc shaped molecules with central holes. The cloned *T. ferrooxidans* GS was regulated by Mg^{2+} or Mn^{2+} , adenylation, and nitrogen source but was not affected by feedback modifiers. The GS has a γ -glutamyltransferase isoactivity point of pH 7.71.

INTRODUCTION

In the nitrogen metabolism of bacteria, glutamine synthetase (GS) (EC 6.3.1.2) plays a central role, as it catalyses one of the main reactions by which ammonia is assimilated (Tyler, 1978):

No studies have been made of GS in strains of the acidophilic autotroph *Thiobacillus ferrooxidans*. *T. ferrooxidans* is the most important bacterium in the leaching of metals from mineral ores, and as the scarcity of nitrogen in leach liquors may limit the efficiency of bacterial leaching operations (Tuovinen *et al.*, 1979), it is important that the enzymes involved in nitrogen metabolism be studied. Investigations on intracellular enzymes in *T. ferrooxidans* are hampered, as it is an obligate autotroph, and high yields of cells are difficult to obtain. To overcome this problem we cloned the *T. ferrooxidans* GS structural gene *glnA*, and nitrogen regulatory genes which complement the *ntrB* and *ntrC* gene functions in *Escherichia coli* (Barros *et al.*, 1985). We describe here the purification and characterization of cloned *T. ferrooxidans* GS produced in *E. coli*.

METHODS

Bacterium and growth conditions. The *E. coli glnA*, *ntrB*, *ntrC* deletion strain ET8051, which harboured the recombinant plasmid pMEB100 containing the *T. ferrooxidans glnA* gene and genes which complement the *ntrB* and *ntrC* gene functions (Barros *et al.*, 1985), was used for the production of GS. *E. coli* ET8051(pMEB100) was grown and maintained in glucose minimal medium containing $(\text{NH}_4)_2\text{SO}_4$ as the sole nitrogen source. In experiments where alternative carbon and nitrogen sources were required, glucose and $(\text{NH}_4)_2\text{SO}_4$, respectively, were replaced by the alternative compounds.

Preparation of cell extracts. All extraction and purification steps were done at 0 to 4 °C with the exception of the heat treatments. For the purification of GS, washed *E. coli* ET8051(pMEB100) cells from 400 ml cultures (OD_{600} 0.9) were resuspended in 20 ml SB buffer (20 mM-imidazole, 10 mM- MgCl_2 , 2 mM-2-mercaptoethanol, pH 7.5)

Abbreviations: GS, glutamine synthetase; γ GT, γ -glutamyl transferase; SVP, snake venom phosphodiesterase.

(approximately 0.19 g wet weight ml⁻¹). The cells were disrupted by two passages through a Yeda Press (Yeda Scientific Instruments, Rehovot, Israel). The preparation was then sonicated and the cell debris removed by centrifugation at 16000 *g* for 1 h. For the experiments on the regulation of GS activity, samples of exponential-phase *E. coli* ET8051(pMEB100) cultures were washed and resuspended in SB buffer. After sonication at 4 °C the cell debris was removed by centrifugation at 16000 *g* for 40 min at 4 °C and the cell extracts were assayed for GS activity.

GS assays. GS activity was measured by the γ -glutamyltransferase (γ GT) assay (total GS activity) of Bender *et al.* (1977) and by the biosynthetic assay of Shapiro & Stadtman (1970). In the biosynthetic assay one unit of GS activity is defined as the amount of enzyme required to form 1 μ mol P_i min⁻¹. Protein was determined by the Lowry procedure.

Purification of cloned *T. ferrooxidans* GS. GS was purified by the method of Streicher & Tyler (1980). The crude extract (20 ml) was centrifuged at 100000 *g* for 1 h and the supernatant (fraction S₁) was re-centrifuged at 100000 *g* for 2 h and the pellet resuspended in 2 ml SB buffer (fraction P₁). The P₁ preparation was kept at 4 °C for 18–24 h and then centrifuged at 12000 *g* for 15 min. The supernatant was centrifuged at 100000 *g* for 2 h and the pellet resuspended in 2 ml SB buffer (fraction P₂). The P₂ fraction was heated at 55 °C for 10 min and then cooled in an ice bath. Denatured proteins were removed by centrifugation at 12000 *g* for 15 min to yield the supernatant fraction S₂.

Polyacrylamide gel electrophoresis (PAGE). The fractions obtained during the purification of GS were subjected to SDS-10% (w/v) PAGE according to the methods of Laemmli (1970) and O'Farrell (1975).

Electron microscopy. Purified GS from *E. coli* ET8051(pMEB100) was fixed with 3% (w/v) glutaraldehyde, stained with 2% (w/v) uranyl acetate and observed with a Zeiss EM109 electron microscope.

Snake venom phosphodiesterase (SVP) digestion of the purified GS. This was done by the method of Shapiro *et al.* (1967).

RESULTS

*Purification and characterization of cloned *T. ferrooxidans* GS*

The cloned *T. ferrooxidans* GS produced in *E. coli* ET8051 by pMEB100 was purified sevenfold with a 24% recovery and a high specific activity (Table 1). SDS-PAGE of the purified GS showed two bands with slightly different mobilities (Fig. 1). The top band is characteristic of unadenylylated GS subunits, and the lower band, with slightly higher mobility, is characteristic of adenylylated GS subunits (Lei *et al.*, 1979). The apparent *M_r* of the unadenylylated GS subunit was approximately 60000 (Fig. 1).

The structure of the purified GS was investigated by electron microscopy (Fig. 2). In some of the molecules a central hole was visible; this is characteristic of the dodecameric assembly of the GS subunits from other bacteria (Frey *et al.*, 1975).

Cloned *T. ferrooxidans* GS purified from *E. coli* ET8051 and partially purified GS from *Vibrio alginolyticus* and *E. coli* were electroblotted (Towbin *et al.*, 1979) from polyacrylamide gels onto nitrocellulose filters. *V. alginolyticus* GS antiserum reacted positively with the cloned *T. ferrooxidans* GS and *E. coli* GS, which provided evidence for the structural similarity between the different GS enzymes. The band obtained for the cloned *T. ferrooxidans* GS was between the bands corresponding to the GS from *E. coli* and *V. alginolyticus*, which is in agreement with the subunit *M_r* values of the GS from these three bacteria (Stadtman & Ginsburg, 1974; Bodasing *et al.*, 1985).

Table 1. *Purification of cloned *T. ferrooxidans* GS from *E. coli* ET8051(pMEB100)*

GS activity was determined by the γ GT assay. The details of the purification procedures and the fractions S₁, P₁, P₂ and S₂ are described in Methods.

Fraction	Vol. (ml)	GS specific activity [μ mol γ -glutamyl hydroxamate (mg protein) ⁻¹ min ⁻¹]	Total protein (mg)	Fold purification	Recovery (%)
Crude extract	20	12.54	80.0	1	100
S ₁	20	8.76	76.7	0.7	67
P ₁	2	29.35	25.0	2	73
P ₂	2	60.02	5.2	5	31
S ₂	2	88.08	2.7	7	24

Fig. 1. SDS-PAGE of crude and purified preparations of cloned *T. ferrooxidans* GS. Lane *a*, crude extract; lane *b*, S_1 fraction; lane *c*, P_1 fraction; lane *d*, P_2 fraction; lane *e*, S_2 fraction; lane *f*, M_r markers. Details of the purification procedure are given in Methods. Protein concentrations applied per lane were: lanes *a*, *b* and *c*, 45 μ g; lanes *d* and *e*, 15 μ g; lane *f*, 30 μ g.

Fig. 2. Electron micrograph of purified cloned *T. ferrooxidans* GS. Bar, 0.01 μ m.

Fig. 3. GS activity from NH_4^+ -shocked (15 mM- NH_4^+) ($\square$, $\circ$) and unshocked ($\blacksquare$, $\bullet$) exponential phase *E. coli* ET8051(pMEB100) cells. (a) Effect of pH on γ GT activity. Cells were NH_4^+ -shocked 7 min before harvesting. (b) Deadenylylated γ GT activity determined by the γ GT assay in the presence of 60 mM- MgCl_2 . The arrow indicates the time of NH_4^+ shocking. SEM were $<5\%$ of the values shown.

NH_4^+ -shocking and isoactivity point determinations

Evidence that the cloned *T. ferrooxidans* GS isolated from *E. coli* contained adenylylated subunits was obtained from SVP digestion and the biosynthetic assay in the presence of Mg^{2+} and Mn^{2+} . Purified GS assayed in the presence of Mg^{2+} and Mn^{2+} showed an activity of 0.68 and 8.26 units (mg protein)⁻¹, respectively. After treatment of this enzyme preparation with SVP, which released all the AMP from the GS, the Mg^{2+} -dependent activity increased to 8.26 units (mg protein)⁻¹, while the Mn^{2+} -dependent activity decreased to 0 units (mg protein)⁻¹.

Since adenylylated and deadenylylated GS from some bacteria have been shown to exhibit different pH response curves (Bender *et al.*, 1977), the isoactivity point at which both forms of the enzyme have the same activity was determined for cloned *T. ferrooxidans* GS. Exponential phase cells of *E. coli* ET8051(pMEB100) in glucose minimal medium were 'shocked' by the addition of $(\text{NH}_4)_2\text{SO}_4$ (15 mM) and harvested 7 min later. Crude cell extracts from shocked and unshocked cultures were assayed for γ GT activity between pH 6.0 and 8.0 (Fig. 3a). The isoactivity point was at pH 7.71 (Fig. 3a).

Further evidence that the cloned *T. ferrooxidans* GS contained adenylylated and deadenylylated subunits was obtained by assaying the deadenylylated form of GS (Mg^{2+} -dependent) after shocking a growing culture of *E. coli* ET8051(pMEB100) with 15 mM- $(\text{NH}_4)_2\text{SO}_4$ (Fig. 3b). Mg^{2+} -dependent γ GT activity decreased to zero 65 min after the addition of $(\text{NH}_4)_2\text{SO}_4$.

Effect of nitrogen source on GS activity

High γ GT specific activity of cloned *T. ferrooxidans* GS [approximately 30 μmol γ -glutamyl hydroxamate (mg protein)⁻¹ min⁻¹] was obtained at low concentrations of $(\text{NH}_4)_2\text{SO}_4$ (0.75 mM), whereas at concentrations of 6 to 45 mM- $(\text{NH}_4)_2\text{SO}_4$, lower γ GT specific activities were obtained [approximately 9 to 11 μmol γ -glutamyl hydroxamate (mg protein)⁻¹ min⁻¹].

The nature of the nitrogen source affected cloned *T. ferrooxidans* GS activity in *E. coli* (Table 2). Growth with proline as the sole nitrogen source markedly enhanced GS activity, whereas GS activity was repressed over 90% by growth with histidine and arginine.

Table 2. Effect of nitrogen source for growth of *E. coli* on the activity of the cloned *T. ferrooxidans* GS enzyme

E. coli ET8051(pMEB100) was grown in succinate minimal medium containing various nitrogen compounds. GS specific activities of mid-exponential phase cells was determined by the γ GT assay. The results are expressed as percentages of the activity in cells grown in succinate minimal medium containing 7.5 mM-(NH₄)₂SO₄, which was 12.54 μ mol γ -glutamyl hydroxamate (mg protein)⁻¹ min⁻¹. The values are means of three determinations; SEM were 5 to 10% of the values shown.

Nitrogen source	Concn (mM)	GS activity (%)
(NH ₄) ₂ SO ₄	7.5	100
Proline	43	253
Histidine	23	6
Arginine	24	4
Glutamine	34	94
Glutamate	34	73

Catabolite repression of GS activity

The addition of glucose or fructose (27 mM) to exponential *E. coli* ET8051(pMEB100) cultures in succinate minimal medium repressed the level of cloned *T. ferrooxidans* GS activity by 20–30%.

Effect of feedback modifiers on cloned *T. ferrooxidans* GS

This was determined in crude extracts prepared from cells grown in glucose minimal medium. The feedback modifiers were added to the γ GT substrate solution before the addition of the crude extract. The γ GT activity of cell-free cloned *T. ferrooxidans* GS was not affected by proline (43 mM), histidine (24 mM), arginine (24 mM), glutamate (34 mM), AMP (35 mM) and (NH₄)₂SO₄ (38 mM).

DISCUSSION

The cloned *T. ferrooxidans* GS subunit has an apparent M_r of approximately 60 000. Assuming that the enzyme is a typical dodecamer, the particle M_r for the undissociated GS would be approximately 720 000. The estimated M_r of the cloned *T. ferrooxidans* GS is higher than that for *E. coli*, *Bacillus subtilis*, *Bacillus stearothermophilus* and '*Bacillus caldolyticus*', which have subunits of M_r 56 000, 50 000, 51 000 and 51 000 respectively (Stadtman & Ginsburg, 1974; Deuel *et al.*, 1970; Wedler & Hoffmann, 1974; Wedler *et al.*, 1980). However, the cloned *T. ferrooxidans* GS has a similar M_r to those of *Bradyrhizobium japonicum* and *V. alginolyticus*, which have an estimated particle M_r of 720 000 and 744 000 respectively and are composed of subunits of M_r 60 000 and 62 000 respectively (Bhandari *et al.*, 1983; Bodasing *et al.*, 1985).

The cross-reactivity obtained between the antiserum prepared against *V. alginolyticus* GS and both cloned GS from *T. ferrooxidans* and *E. coli* GS extends the finding that GSs from heterotrophic Gram-negative bacteria are related (Tronick *et al.*, 1973). This study shows that the antigenic relatedness between the GSs of Gram-negative bacteria holds even for an autotrophic chemolithotroph such as *T. ferrooxidans*.

Previously we reported that no homology could be detected between either cloned or chromosomal GS DNA from *E. coli* and cloned GS from *T. ferrooxidans* (Barros *et al.*, 1985). In spite of this lack of homology at the DNA level the GSs from these two bacteria, as determined by both electron microscopy and antigenicity, are related. The GS DNA sequence, therefore, does not appear to be subject to the strong evolutionary constraints seen for the genes of the nitrogenase enzymes (Ruvkun & Ausubel, 1980). The DNA sequence of the *T. ferrooxidans* GS gene should, therefore, provide an important contribution to an understanding of the preferred codon usage of the bacterium.

The *E. coli* GS has been shown to be regulated by at least four different mechanisms: by kinetic factors, including the concentration of the divalent cations Mg²⁺ or Mn²⁺, which are

required for the structural stabilization of the enzyme; by covalent modification of the enzyme by adenylation and deadenylation; by repression and derepression of enzyme synthesis in response to the nitrogen source; and by cumulative feedback inhibition by various end-products of glutamine metabolism (Tyler, 1978; Brown, 1980). Cloned *T. ferrooxidans* GS activity in *E. coli* was regulated by Mg^{2+} or Mn^{2+} , adenylation, and nitrogen source, but was not affected by feedback modifiers which affect *E. coli* and other prokaryotic GSs (Bodasing *et al.*, 1985). GS from *Anabaena* and *Chloroflexus* have also been reported to be resistant to most of the feedback inhibitors that affect many prokaryotic GSs (Kaulen & Klemme, 1983).

Production of the cloned *T. ferrooxidans* GS in *E. coli* was relatively insensitive to regulation by glucose or fructose catabolite repression. Since *T. ferrooxidans* is an obligate autotroph and its growth is inhibited by traces of galactose in agar (Tuovinen & Kelly, 1973), it is perhaps not surprising that the production of GS is not very sensitive to regulation by sugars.

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