JOURNAL OF BACTERIOLOGY, Nov. 1984, p. 591–599
0021-9193/84/110591-09$02.00/0

Vol. 160, No. 2

Purification and Properties of Glutamate Synthase from *Bacillus licheniformis*

HAROLD J. SCHREIER† AND ROBERT W. BERNLOHR*

Biochemistry Program, The Pennsylvania State University, University Park, Pennsylvania 16802

Received 21 May 1984/Accepted 3 August 1984

Glutamate synthase [L-glutamate:NADP$^+$ oxidoreductase (transaminating); EC 1.4.1.13] (GltS) was purified to homogeneity from *Bacillus licheniformis* A5. The native enzyme had a molecular weight of approximately 220,000 and was composed of two nonidentical subunits (molecular weights, ~158,000 and ~54,000). The enzyme was found to contain 8.1 ± 1 iron atoms and 8.1 ± 1 acid-labile sulfur atoms per 220,000-dalton dimer. Two flavin moieties were found per 220,000-dalton dimer, with a ratio of flavin adenine dinucleotide to flavin mononucleotide of 1.2. The UV-visible spectrum of the enzyme exhibited maxima at 263,380 and 450 nm. The GltS from *B. licheniformis* had a requirement for NADPH, α-ketoglutarate, and glutamine. Classical hyperbolic kinetics were seen for NADPH affinity, which resulted in an apparent K_m value of 13 μM. Nonhyperbolic kinetics were obtained for α-ketoglutarate and glutamine affinities, and the reciprocal plots obtained for these substrates were biphasic. The apparent K_m values obtained for glutamine were 8 and 100 μM, and the apparent K_m values obtained for α-ketoglutarate were 6 and 50 μM. GltS activity was found to be relatively insensitive to inhibition by amino acids, keto acids, or various nucleotides. L-Methionine-DL-sulfoximine, L-methionine sulfone, and DL-methionine sulfoxide were found to be potent inhibitors of GltS activity, yielding $I_{0.5}$ values of 150, 11, and 250 μM, respectively. GltSs were purified from cells grown in the presence of ammonia and nitrate as sole nitrogen sources and were compared. Both yielded identical final specific activities and identical physical (UV-visible spectra, flavin, and iron-sulfur composition) and kinetic characteristics.

In most procaryotes ammonia is assimilated via the combined action of glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming); EC 6.3.1.2] (reaction i) and glutamate synthase [L-glutamate:NADP$^+$ oxidoreductase (transaminating); EC 1.4.1.13] (GltS; reaction ii), as follows:

$$\text{glutamate} + \text{ATP} + \text{NH}_3 \rightarrow \text{glutamine} + \text{ADP} + \text{P}_i \quad \text{(i)}$$

$$\text{glutamine} + \alpha\text{-ketoglutarate} + \text{NADPH} + \text{H}^+ \rightarrow 2(\text{glutamate}) + \text{NADP}^+ + \text{H}_2\text{O} \quad \text{(ii)}$$

Glutamate dehydrogenase [L-glutamate:NADP$^+$ oxidoreductase (deaminating); EC 1.4.1.4) (GDH; reaction iii) has also been shown to play a role in the assimilation of ammonia in some organisms under certain physiological conditions (36, 46).

$$\alpha\text{-ketoglutarate} + \text{NH}_3 + \text{NADPH} + \text{H}^+ \rightarrow \text{glutamate} + \text{NADP}^+ \quad \text{(iii)}$$

Most studies on the regulation of activity of the enzymes of ammonia assimilation have utilized the enteric organisms (24, 25, 46). It is becoming increasingly clear that other organisms use control systems for nitrogen metabolism and assimilation which may be different from those described for the enteric bacteria (7, 30, 40). For instance, the glutamine synthetase from *Escherichia coli* and *Klebsiella aerogenes* is controlled via a complex covalent modification system (25, 33, 36), wherease the *Bacillus licheniformis* and *Bacillus subtilis* glutamine synthetases are regulated primarily by feedback inhibition (9, 11).

An understanding of the factors involved in the regulation of nitrogen metabolism in the *Bacillus* spp. may yield valuable information regarding the nature of the signals which trigger vegetative cells to sporulate under nitrogen limitation conditions. Characterization of both the glutamine synthetase and the GDH from *B. licheniformis* have been described previously (10, 11, 35), whereas an extensive study of the GltS from a *Bacillus* sp. has not been reported previously.

GltS has been purified to homogeneity from *E. coli* (32), *K. aerogenes* (45), *Bacillus megaterium* (16), and *Saccharomyces cerevisiae* (31, 39; D. S. Masters, Jr., and W. B. Rowe, Fed. Proc. 38:724, 1979). This enzyme has been shown to be an iron-sulfur flavoprotein which consists of a dimer of two unequal subunits. Unlike other amidotransferases, the large subunit of GltS binds glutamine and catalyzes the following glutaminase reaction (13, 26, 27, 45):

$$\text{glutamine} + \text{H}_2\text{O} \rightarrow \text{glutamate} + \text{NH}_3 \quad \text{(iv)}$$

The ammonia transfer reaction is catalyzed by the small subunit (13, 26, 28, 45), as follows:

$$\text{NH}_3 + \alpha\text{-ketoglutarate} + \text{NADPH} + \text{H}^+ \rightarrow \text{glutamate} + \text{NADP}^+ \quad \text{(v)}$$

In this report we describe the purification and properties of the GltS from *B. licheniformis*. We found this enzyme to be similar in some respects to the enzymes from other sources. However, the GltS from *B. licheniformis* was found to exhibit unique spectral, amino acid composition, kinetic, and regulatory properties which have not been previously described for this enzyme.

MATERIALS AND METHODS

Growth and harvesting of *B. licheniformis*. *B. licheniformis* A5 was grown in the minimal salts A medium previously described (42) with the modifications described by Donohue and Bernlohr (10). Unless otherwise indicated, glucose (15 mM) and ammonium sulfate (10 mM) served as carbon and

* Corresponding author.
† Present address: Department of Molecular Biology and Microbiology, Tufts University, Boston, MA 02111.

nitrogen sources. Other carbon and nitrogen sources were used at the concentrations indicated below.

Cultures were harvested at one generation before the initiation of the stationary phase of growth, as described previously (10). Sedimented cells were washed (41) and used immediately or, when the cells were used for GltS purification, were stored at −70°C.

Preparation of buffers. TME buffer contained 25 mM Tris-hydrochloride (pH 7.9), 10 mM β-mercaptoethanol, 1 mM Na_2EDTA, and 2 mM α-ketoglutarate. TMEG buffer was identical to TME buffer but, in addition, contained 10% glycerol. IME buffer contained 25 mM imidazole hydrochloride (pH 6.8), 10 mM β-mercaptoethanol, 1 mM Na_2EDTA, and 2 mM α-ketoglutarate. IMEG buffer was identical to IME buffer but, in addition, contained 10% glycerol. IDEG buffer, which was used for storage of the purified enzyme, contained 25 mM imidazole hydrochloride (pH 7.5), 10 mM dithiothreitol, 1 mM Na_2EDTA, and 20% glycerol. All buffers were boiled before the addition of β-mercaptoethanol, dithiothreitol, and α-ketoglutarate to remove dissolved oxygen and were cooled to 4°C under a stream of oxygen-free nitrogen.

Preparation of cell extracts. Cells from 1 liter of medium were suspended in 4 ml of TME buffer, and cell extracts were prepared as described previously (41). For the preparation of crude cell extracts to be used for the purification of GltS, cells were suspended at a ratio of 1 g (wet weight) of cells per 2 ml of IME buffer containing 1 mM phenylmethylsulfonyl fluoride. The cells were disrupted as described above, DNase (EC 3.1.4.5) was added to a concentration of 5 μg/ml, and the resulting preparation was stirred slowly at 4°C for 1 h. Debris was removed by centrifugation at 40,000 × *g* for 30 min (4°C). The supernatant fraction was designated "crude" and yielded protein concentrations in the range of 25 to 30 mg/ml. Protein was assayed by the 230/260 method of Kalb and Bernlohr (20).

Enzyme assays. GltS and GDH were assayed by recording the rate of oxidation of NADPH at 340 nm, using a Cary model 118 recording spectrophotometer. GltS was assayed as described previously (41). The ammonia-dependent reaction of GltS was assayed by substituting ammonium chloride (100 mM) for glutamine. GDH was assayed as described by Phibbs and Bernlohr (35). One unit of activity for both GltS and GDH corresponds to the amount of enzyme catalyzing the utilization of 1 μmol of NADPH per min unless otherwise stated. Glucose-6-phosphate dehydrogenase was assayed by recording the rate of $NADP^+$ reduction at 340 nm as described by Miller and Stadtman (32). One unit of activity was defined as the formation of 1 μmol of NADPH per min. NADPH concentrations were determined by using a molar absorbtivity of 6.22×10^3. Glutamine synthetase was assayed by the γ-glutamylhydroxamate assay of Hunt et al. (17).

All assays were done at 37°C unless otherwise noted. Additional details on the characteristics of the above activities in *B. licheniformis* extracts have been reported elsewhere (Schreier, Ph.D. thesis, The Pennsylvania State University, University Park, 1983).

Purification of GltS. All steps were performed at 0 to 5°C, and all centrifugations were at 40,000 × *g* for 20 min, unless otherwise stated. Streptomycin sulfate was added to crude extract to a final concentration of 2% (wt/vol), and the pH of the preparation was brought to 6.0 with 1 N acetic acid. After stirring and centrifugation, the supernatant fraction was brought to 50% (vol/vol) ammonium sulfate by using an equal volume of a saturated ammonium sulfate solution (800 g of ammonium sulfate per liter of IME buffer), and the pH was maintained at 6.0 by the addition of 1 N acetic acid. The cloudy preparation was allowed to stir slowly for 1 h and was then centrifuged. The supernatant fraction was brought to 67.5% (vol/vol) ammonium sulfate by the addition of 0.5 ml of saturated ammonium sulfate per ml of preparation, stirred for 1 h, and centrifuged. The dark yellow sedimented fraction was dissolved in minimal amounts of TMEG buffer which contained 100 mM KCl.

The dissolved 50 to 67.5% ammonium sulfate fraction was dialyzed for 16 h against 100 volumes of TMEG buffer containing 100 mM KCl. The dialyzed material was applied to a DEAE-Sephacel column which had been equilibrated with TMEG buffer containing 100 mM KCl and washed with 2 column volumes of the same buffer. Protein was eluted from the column by using a 1.5-liter linear gradient of 100 to 400 mM KCl in TMEG buffer. As shown in Fig. 1, the GltS activity and associated 440-nm-absorbing material eluted approximately halfway through the gradient. GDH activities were found at two places, one corresponding to the GltS fraction and another eluting 9 to 10 fractions later. This latter activity was shown previously (35) to be a typical ammonia-dependent GDH. The GltS-containing fractions were concentrated to a volume of 5 ml via ultrafiltration by using an Amicon ultrafiltration apparatus equipped with a PM10 membrane under an atmosphere of oxygen-free nitrogen.

The concentrated protein was added to a Bio-Gel A 1.5m column (200 to 400 mesh) which had been equilibrated with IMEG buffer and eluted with the same buffer. GltS activity, with the associated 440 nm absorption and GDH activity, eluted from the column within 10 to 20 fractions of the void volume of the column.

The fractions containing the majority of the GltS activity were pooled and applied to a Red Sepharose CL-6B column equilibrated with IMEG buffer. Nonbinding protein was eluted from the column by washing with IMEG buffer, and GltS was eluted with IMEG buffer containing 2 mM glutamine and 250 μM NADPH. Upon elution it was observed that the 440-nm-absorbing material remained associated with the GltS-containing fractions. After the fractions were pooled and concentrated by ultrafiltration, the preparation was passed through a Sephadex G-25 column equilibrated with IMEG buffer to remove glutamine and NADPH and dialyzed against IDEG buffer to remove α-ketoglutarate. The final preparation was stored in IDEG buffer at −20°C.

In the presence of IDEG buffer, purified GltS had a half-life of 24 h at 25°C, 3 to 4 days at 4°C, and 1 month at −20°C. Storage at −70°C did not improve enzyme stability, and neither did inclusion of substrates in the buffer, a condition which aided the stability of GltSs from other sources (16, 32, 45).

A summary of a typical purification procedure for GltS isolated from cells grown with ammonia as a nitrogen source is presented in Table 1. This procedure was repeated five times with similar results. The specific activity of purified GltS was 22.9 U/mg of protein, which represented a 63.6-fold purification with a 25% yield. The ammonia-dependent activity in the final preparation was 0.65 U/mg of protein and was 2.8% of the final activity of GltS.

Using the same procedure, the purification of GltS from cells grown in the presence of nitrate as the nitrogen source yielded a preparation which gave a final specific activity of 23.1 U/mg. This represented a 91.0-fold purification with a 22% yield. Identical specific activities, yields, and chromatographic elution patterns were obtained from both preparations.

Since comparison studies were performed by using the *B. licheniformis* GDH, a partially purified preparation was obtained by utilizing the procedure for the purification of GltS. The material sedimenting in 50% (vol/vol) ammonium sulfate was suspended in TMEG buffer containing 100 mM KCl and dialyzed against the same buffer to remove residual ammonium sulfate. This preparation was added to the DEAE-Sephacel column, and chromatography was accomplished as described above for GltS. Subsequent purification procedures were the same as for GltS, and the GDH preparation obtained after the Red Sepharose affinity chromatography step had a specific activity of 35.9 U/mg of protein, representing a 133-fold purification and a 31.1% yield. This preparation was not homogeneous, as judged by polyacrylamide gel electrophoresis; however, only one protein contaminant was detected (data not shown).

Electrophoresis. Disc gel electrophoresis was performed at 4°C by modification of the method described by Orr et al. (34). The triethanolamine-TES buffer system was used in conjunction with 6.25% acrylamide separating gels and 2.5% acrylamide stacking gels. Glycerol was added to the separating gel at a final concentration of 25%. Both stacking and separating gels were polymerized with ammonium persulfate (0.63 mg/ml, final concentration). When this system was used, GltS mobility was retarded compared with GDH. Identification of the location of both activities was accomplished by incubating smashed slices (1.0 mm) in IDEG buffer for 18 h and assaying for activity.

Electrophoresis under denaturing conditions was done by using a modification of the procedure of Weber and Osborn (47) and Davies and Stark (8), as described in Sigma Chemical Co. *Bulletin MWS-877* (43). A 5% acrylamide gel was used in conjunction with the sodium dodecyl sulfate-sodium phosphate buffer system. The proteins (and their respective molecular weights) used for the estimation of the *B. licheniformis* GltS subunit molecular weights were cross-linked hemocyanin (70,000, 140,000, 200,000, and 280,000), cross-linked albumin (66,000, 132,000, 198,000, and 264,000), and ovalbumin (43,000).

GltS cross-linking studies. Amidination of GltS were performed in 0.2 M triethanolamine hydrochloride (pH 8.5) as described by Davies and Stark (8). The final concentrations of GltS and dimethyl suberimidate were 0.9 and 3.3 mg/ml, respectively. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis system was performed as described above, using a 3.5% gel.

Sucrose density gradient sedimentation. Sedimentation analyses of GltS and GDH were done as described by Martin and Ames (29). GltS and GDH were prepared by dialyzing against 1,000 volumes of 25 mM Tris-hydrochloride (pH 7.9)–10 mM β-mercaptoethanol–1 mM Na_2EDTA to remove glycerol. Sucrose gradients were prepared in a buffer containing 25 mM Tris-hydrochloride (pH 7.9), 10 mM β-mercaptoethanol, 1 mM Na_2EDTA, and 10 mM $MgCl_2$. Glucose-6-phosphate dehydrogenase (molecular weight, 101,600; sedimentation coefficient corrected to water at 20°C [$s_{20,w}$], 20.3) and purified *E. coli* glutamine synthetase (molecular weight, 600,000; $s_{20,w}$, 20.3) (32) were used as reference standards. The purified glutamine synthetase was a gift from F. C. Wedler.

Determination of iron, molybdenum, and flavin. The iron content of purified GltS was determined by a modification of the method described by Lovenberg et al. (23). GltS (0.1 mg in 0.25 ml of IDEG buffer) was added to 1.25 ml of 1% HCl. This mixture was heated to 80°C for 10 min and centrifuged, and the supernatant fraction was transferred to a 3-ml curvette. Then 1 ml of 0.3% 1,10-phenanthroline and 0.5 ml of 0.2 M potassium biphthalate were added, the preparation was mixed, and the absorbance was measured at 512 nm. IDEG buffer was used as a blank.

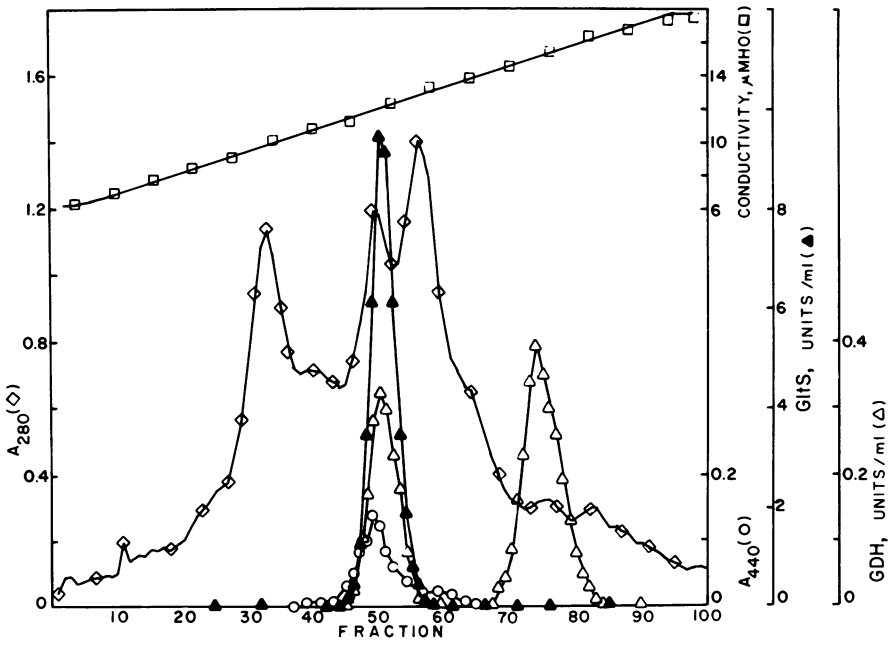

FIG. 1. Elution pattern of *B. licheniformis* GltS activity on a DEAE-Sephacel column (2.5 by 65 cm). GltS (▲) and GDH (△) were assayed under standard conditions (see text). Protein was monitored by absorbance at 280 nm (A_{280}) (◇), and absorbance at 440 nm (○) was also monitored. Conductivity (□) was measured by using a Yellow Springs model 31 conductivity bridge. Elution was done at a flow rate of 0.5 ml/min, and 9.8-ml fractions were collected.

TABLE 1. GltS purification summary[a]

Step	Prepn or treatment	Protein (mg)	Units (μmol/min)[b]	Sp act (U/mg)	% Yield	Fold purification
I	Crude extract	2,738	986	0.36	100	1.0
II	Streptomycin sulfate	2,220	866	0.39	88	1.1
III	$(NH_4)_2SO_4$ precipitation	650	631	0.97	64	2.7
IV	DEAE-Sephacel	96	478	5.0	49	13.8
V	Bio-Gel A 1.5 m	55	429	7.8	44	21.7
VI	Red Sepharose CL-6B and Sephadex G-25	10.8	274	22.9	25	63.6

[a] Purified from 50 g (wet weight) of frozen cell paste prepared from cells grown in the presence of glucose and ammonia and harvested one generation before the end of exponential growth as described in the text.
[b] GltS was assayed under standard conditions as described in the text.

Molybdenum was determined by the method of Johnson and Arkley (18). Total flavin was determined spectrophotometrically by the hydrosulfite procedure of Rao et al. (37). An extinction coefficient of $9.8 \times 10^3\ M^{-1}\ cm^{-1}$ was used to determine total flavin. The ratios of flavin adenine dinucleotide to flavin mononucleotide were determined by the fluorometric method of Yagi (48). GltS (2.1 mg) was dialyzed against 1,000 volumes of 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–KOH buffer (pH 7.5), 1 mM Na_2EDTA, and 5 mM β-mercaptoethanol to remove glycerol and fluorescing imidazole.

Determination of acid-labile sulfur. Acid-labile sulfur was determined to a modification of the methylene blue procedures described by King and Morris (21) and Helmerhost and Stokes (15). GltS preparations were dialyzed against 1,000 volumes of 25 mM imidazole hydrochloride (pH 7.5) and 1 mM Na_2EDTA to remove dithiothreitol. GltS (18 μg in 0.35 ml), 0.25 ml of 2.6% zinc acetate, and 0.05 ml of 6% NaOH were sealed in a 1.0-ml Reacti-vial. After gentle mixing, 0.1 ml of 20 mM *N,N*-dimethyl-*p*-phenylenediamine in 7.2 M HCl was injected into the vial. After gentle swirling until the samples cleared, 0.05 ml of 30 mM ferric chloride in 1.2 M HCl was injected into the vial, and the vial was mixed and allowed to stand for 30 min in the dark. The samples were transferred to centrifuge tubes containing 0.425 ml of water, mixed, and centrifuged, and the absorbance of the supernatant fraction was measured at 668 nm. An extinction coefficient of $25.3 \times 10^3\ M^{-1}\ cm^{-1}$ was used to calculate sulfide concentrations (15).

Amino acid analysis. Amino acid analysis was carried out by AAA Laboratories, Mercer Island, Wash., using a standard 24-h hydrolysis in 6 N HCl with corrections for losses of threonine (+5%) and serine (+10%). Tryptophan was determined by the procedure of Edelhoch (12), and half-cystines were determined as described by Habeeb (14).

Determination of $NADP^+$ and NADPH pools. Cell extracts for the determination of $NADP^+$ and NADPH pools were prepared as described previously (41), except that filtered cells were extracted either in 0.3 N $HClO_4$ for the determination of $NADP^+$ (22) or in 0.2 M KOH for the determination of NADPH (22). $NADP^+$ and NADPH were assayed in these extracts by the spectrophotometric cycling assay of Jorgensen and Rasmussen (19). Millimolar intracellular concentrations were calculated as described previously (42).

Materials. Bio-Gel A 1.5 m was obtained from Bio-Rad Laboratories, Richmond, Calif. DEAE-Sephacel, Sephadex G-25, and Red Sepharose CL-6B were obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Silica gel was from Eastman Corp., Rochester, N.Y. Enzyme grade, "ultrapure" ammonium sulfate was from Schwarz/Mann, Orangeburg, N.Y. DNase (beef pancreas; type DN-100), glucose-6-phosphate dehydrogenase (yeast; type XI), catalase (*Aspergillus niger*; dialyzed to remove ammonium sulfate), and all other biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of reagent grade. Double-distilled water was used to prepare all solutions. Nitrogen gas which contained less than 1 μl of oxygen per liter was obtained from Matheson Scientific, Inc., East Rutherford, N.J.

RESULTS

Homogeneity and properties of purified GltS. The purified enzyme was judged to be homogeneous on the basis of polyacrylamide gel electrophoresis. All detectable GltS activity was associated with a single protein band observed in stained gels. Electrophoresis of partially purified GltS preparations showed that contaminating GDH activity migrated faster than GltS activity (data not shown).

GltS was denatured with sodium dodecyl sulfate, and electrophoresis was performed as described above. The gel showed that the GltS from *B. licheniformis* consisted of two nonidentical subunits and thus was similar to the GltSs studied for all other procaryotes. A comparison of the relative mobilities of the purified GltS subunits compared with the relative mobilities of the standard proteins (see above) indicated that the *B. licheniformis* GltS subunit molecular weights were 54,000 ± 2,000 and 158,000 ± 5,000.

To determine the molecular weight of the native form of the enzyme, purified GltS was sedimented in a sucrose density gradient at 37,000 rpm. By using glutamine synthetase and glucose-6-phosphate dehydrogenase as standards, an $s_{20,w}$ of approximately 10.2, corresponding to a molecular weight of 220,000 ± 7,000, was obtained. This value indicated that the GltS from *B. licheniformis* was a dimer composed of two nonidentical subunits. The sedimentation profile was identical when $MgCl_2$ and β-mercaptoethanol were eliminated from the buffer. The presence of either 400 mM KCl, 2 mM α-ketoglutarate, 2 mM glutamine, or 0.2 mM NADPH (with or without glutamine or α-ketoglutarate) did not alter the molecular weight of the enzyme. The sedimentation of a crude extract at a protein concentration of 3.3 mg/ml also yielded an identical pattern. Increasing the protein concentration of purified GltS threefold did not change the molecular weight observed for the native enzyme. We concluded that conditions which resulted in the aggregation of the GltSs from other organisms did not cause aggregation of the GltS from *B. licheniformis* to form active higher-molecular-weight species (32, 49). For comparison, the GDH from *B. licheni-*

formis was found to have a molecular weight of 310,000 ± 9,000.

To determine whether the GltS from *B. licheniformis* could aggregate to form higher-molecular-weight species, amidination in the presence of dimethyl suberimidate was performed as described by Davies and Stark (8). Three protein bands were obtained when the reaction mixture was denatured and separated on 3.5% polyacrylamide gels; these three bands were calculated to have molecular weights of 54,000, 158,000, and 217,000. No higher-molecular-weight species were found when longer incubation periods were used.

The UV-visible spectrum of the purified *B. licheniformis* GltS exhibited absorption maxima at 263, 380, and 450 nm (data not shown). Absorbance at 450 nm is indicative of enzymes which contain flavins (48). Addition of 1 mM sodium dithionite to the enzyme preparation yielded a decrease in the absorption at 380 and 450 nm, which is characteristic of dithionite reduction of GltSs from other organisms (4, 13, 28, 32). The addition of 250 μM NADPH caused a decrease in the absorbance at 450 nm. The maximum seen at 263 nm is a unique characteristic of the GltS from *B. licheniformis*, as it replaces the maximum observed at 280 nm of other GltSs (13, 32).

The fluorescence spectrum of purified GltS showed emissions at 340 nm (exciting at 280 nm) and 530 nm (exciting at 430 nm). Denaturation of the enzyme by addition of 10% sodium dodecyl sulfate increased the fluorescence intensity of the 530-nm emission maximum and shifted that at 340 nm to 320 nm. An analysis of the GltS purified from cells grown in the presence of nitrate revealed similar UV-visible and fluorescence spectra.

Duplicate analyses of three different preparations of GltS yielded a total flavin composition of 2.1 ± 0.4 mol per 220,000-molecular-weight species. This value was similar to those obtained for the GltSs isolated from other sources (32, 45). Using the fluorescence assay described above, we determined that the ratio of flavin adenine dinucleotide to flavin mononucleotide was 1.2. The presence of both flavin adenine dinucleotide and flavin mononucleotide appears to be common among all GltSs studied. Similar results were obtained when we analyzed the GltS purified from cells grown in the presence of nitrate.

Triplicate analyses of two different preparations showed that the GltS from *B. licheniformis* contained 8.1 ± 1 iron atoms and 8.1 ± 1 acid-labile sulfur atoms per 220,000-molecular-weight species. These values were found to be similar to the values for iron and acid-labile sulfur obtained by other workers for GltS (32, 45). Similar results were obtained for the iron and sulfur composition of the GltS purified from cells grown in the presence of nitrate. Molybdenum was not detected.

The amino acid composition of the GltS isolated from *B. licheniformis* is shown in Table 2, where it is compared with previously published analyses of the enzymes from *B. megaterium* and *E. coli* (16, 32). The amino acid composition for the *B. licheniformis* GltS appeared to be different from the compositions of the enzymes from the other two sources, indicating that the enzyme from *B. licheniformis* is a relatively different GltS.

GltS catalytic requirements. The apparent pH optimum for the glutamine-dependent activity of GltS was found to be 7.8, whereas the ammonia-dependent activity exhibited a pH optimum greater than 8.7. Both of these values were similar to pH optima obtained by other workers for the glutamine- and ammonia-dependent activities of GltS (13, 28, 32, 38).

TABLE 2. Amino acid compositions of GltS from various organisms[a]

Residue	Amt in GltS from:		
	B. licheniformis[b]	*B. megaterium*[c]	*E. coli*[d]
Alanine	180	156	149
Arginine	98	100	97
Asx	193	175	165
Cystine/2	16	17	18
Glx	214	177	157
Glycine	203	162	149
Histidine	54	33	30
Isoleucine	118	74	76
Leucine	162	167	142
Lysine	123	72	75
Methionine	8	34	36
Phenylalanine	93	59	58
Proline	78	71	71
Serine	127	82	70
Threonine	124	86	74
Tryptophan	18	NR[e]	18
Tyrosine	60	35	42
Valine	132	106	109

[a] Values were rounded to the nearest whole residue.
[b] Data from this study. See test for details. Values are expressed as moles per 220,000-dalton dimer.
[c] Data from reference 16. Values are expressed as moles per 210,000-dalton dimer.
[d] Data from reference 32. Values are expressed as moles per 200,000-dalton dimer.
[e] NR, Not reported.

GDH was found to have a broad pH profile, with an apparent pH optimum between 7.8 and 8.0. These values indicated that the *B. licheniformis* ammonia-dependent activity of GltS was not characteristic of the GDH from *B. licheniformis* (35).

The effects of KCl, NaCl, $CaCl_2$, and $MnCl_2$ on GltS activity were examined. KCl and NaCl had no effect on GltS activity. $CaCl_2$, $MgCl_2$, and $MnCl_2$ at concentrations of 50 mM inhibited GltS activity 27, 31, and 64%, respectively. A similar study using the GltS from *E. coli* assayed under similar conditions showed that the *E. coli* was more sensitive to inhibition by these cations (32). The high concentrations of cations required to inhibit the *B. licheniformis* GltS indicated that this inhibition was probably not physiologically significant.

L-Glutamine, α-ketoglutarate, and NADPH were required for catalytic activity. In the absence of both α-ketoglutarate and L-glutamine or with only α-ketoglutarate present, the purified enzyme displayed negligible NADPH oxidase activity. NADH (250 μM) did not support activity. L-Asparagine, D-asparagine, or γ-aminobutyric acid at a final concentration of 20 mM could not replace glutamine. *N*-acetyl-L-glutamine at a concentration of 100 mM supported approximately 6% of the control activity, but this activity could have been due to contamination by glutamine. α-Ketobutyrate, α-ketovalerate, α-ketoisovalerate, α-ketocaproate, ketomalonate, oxaloacetate, or pyruvate (at a concentration of 35 mM) could not substitute for α-ketoglutarate in the reaction.

Kinetic constants for GltS substrates. GltS showed classical Michaelis-Menten kinetics with respect to NADPH. A representative reciprocal plot is shown in Fig. 2A. The apparent K_m value for NADPH was calculated to be 13 μM. This value was similar to the values obtained for other GltSs (1, 16, 32, 38, 45, 49) and was the same for the enzyme isolated from cells grown in the presence of nitrate as the nitrogen source.

Reciprocal plots obtained for glutamine and α-ketoglutar-

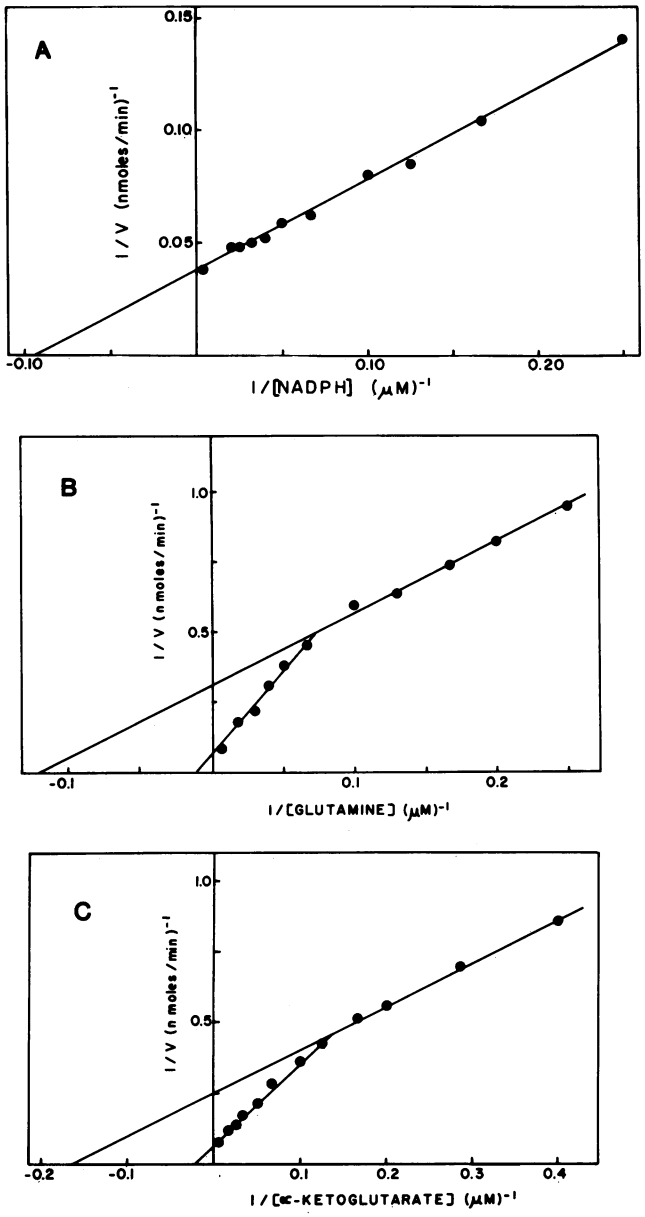

FIG. 2. Effect of substrate concentration on reaction velocity. GltS (2.0 μg/ml) was assayed under standard conditions except for the modifications described below. (A) NADPH concentration was varied, and the concentrations of glutamine and α-ketoglutarate were 10 mM. (B) Glutamine concentration was varied, and the concentration of α-ketoglutarate was 10 mM. (C) α-Ketoglutarate concentration was varied, and the concentration of glutamine was 10 mM. Each point is the average of duplicate assays.

ate saturation are shown in Fig. 2B and C, respectively. Both exhibited nonhyperbolic kinetics, a phenomenon which has not been described for the GltS characterized from other sources (1, 16, 32, 38, 39, 45, 49). The best-fit lines yielded apparent K_m values of approximately 8 and 100 μM for glutamine and 6 and 50 μM for α-ketoglutarate. Similar patterns were obtained for the GltS purified from either ammonia- or nitrate-grown cells. Inclusion of 1 mM Na_2EDTA or deletion of β-mercaptoethanol from the assay mixture did not alter the biphasic nature of these patterns.

Inhibition of enzyme activity. Various compounds were tested for their effects on GltS activity. GltS activity was inhibited by 5 mM L-methionine, L-leucine, L-serine, L-histidine, L-glycine, or L-analine (16, 16, 10, 5, 5, and 5%, respectively), when GltS was assayed under standard conditions but with 0.5 mM glutamine and 0.5 mM α-ketoglutarate in the assay mixture. Further examination of the effects of these amino acids on GltS activity showed that combinations of these compounds acted in a cumulative manner when they were tested at concentrations of 5 mM each (data not shown). The maximum inhibition obtained when all of the above amino acids were combined (at concentrations of 5 mM each) was only 38%, and this was not considered to be physiologically significant.

The compounds tested which had no detectable effect on enzyme activity when added to a standard GltS assay mixture at concentrations of 5 and 25 mM were D-aspartate, D-asparagine, D-methionine, L-tryptophan, L-proline, L-isoleucine, L-arginine, L-phenylalaine, L-lysine, L-threonine, γ-aminobutyric acid, NAD^+ (tested at 1 mM), carbamyl phosphate, acetyl phosphate, acetyl coenzyme A, coenzyme A, aminooxyacetate, malate, oxalacetate, succinate, isocitrate, pyrophosphate, phosphate, phosphoribosylpyrophosphate, glucosamine, glucosamine-6 phosphate, ribose-5 phosphate, AMP, ADP, ATP, CMP, CDP, CTP, GMP, GTP, UMP, UDP, and ITP. Similar results were obtained when GltS was assayed under standard conditions by using glutamine and α-ketoglutarate concentrations of either 0.5 or 0.025 mM.

When GltS was assayed in the presence of 0.5 mM glutamine, 0.5 mM α-ketoglutarate, and 250 μM NADPH, we found that the L-glutamine analogs L-methionine-DL-sulfoximine, L-methionine sulfone, and DL-methionine sulfoxide were potent inhibitors, yielding $I_{0.5}$ values of 150, 11, and 250 μM, respectively (data not shown).

$NADP^+$ was found to be a potent inhibitor of GltS activity, yielding 50% inhibition when the ratio of $NADP^+$ to NADPH was 2:1 (over an $NADP^+$ concentration range of 20 to 500 μM). The reciprocal plots obtained for the inhibition of GltS activity by varying concentrations of $NADP^+$ with respect to NADPH showed that the inhibition of activity by $NADP^+$ was competitive with respect to NADPH (data not shown), and an apparent K_i value for $NADP^+$ of 20 μM was determined.

To determine whether the inhibition of GltS activity by $NADP^+$ may be physiologically significant, intracellular concentrations of $NADP^+$ and NADPH were measured in growing and sporulating cultures of *B. licheniformis* grown in the presence of glucose and ammonia. Samples were taken 1 h before the onset of stationary phase, at the beginning of stationary phase, and 2 h after the start of stationary phase. The $NADP^+$ pools were 18, 66, and 20 μM, respectively, and the NADPH pools were 120, 195, and 65 μM, respectively. In no instance did the ratio of $NADP^+$ to NADPH approach 2:1, and we therefore concluded that under these conditions GltS was probably not significantly regulated by the $NADP^+$ pool.

Inactivation of GltS by NADPH. Many investigators have shown that GltSs from various sources, when incubated in the presence of NADPH without other substrates, become irreversibly inactivated (13, 27, 32). We found that incubation of NADPH with GltS resulted in a loss in absorbance at 450 nm. To test for the inactivation of the enzyme by NADPH, GltS was treated with NADPH after dialysis against 25 mM imidazole hydrochloride (pH 7.5), 1 mM β-mercaptoethanol, 1 mM Na_2EDTA, and 10% glycerol to remove IDEG buffer components. Inactivation of GltS oc-

curred in the presence of 250 μM NADPH (90% after 60 min). Glutamate, $NADP^+$, or Na_2EDTA did not protect the enzyme from inactivation. Lack of protection by Na_2EDTA was contrary to the results of Mantsala and Zalkin (27) for the *E. coli* enzyme. Protection from inactivation was seen when glutamine, α-ketoglutarate, dithiothreitol, or catalase was added to the reaction mixture. The addition of α-ketoglutarate or glutamine to inactivated enzyme did not result in the restoration of activity.

These results showed that NADPH inactivation of the GltS from *B. licheniformis* did not occur and that protection from inactivation was accomplished by most of the compounds which aided in GltS protection from other sources. The physiological significance of GltS inactivation by NADPH is questionable since glutamine pools have been shown to remain fairly constant (2 to 6 mM) throughout growth under most conditions studied (41), although α-ketoglutarate pools have been shown to drop to low levels (200 μM) (41).

Control of enzyme synthesis. Work done in our laboratory has shown that GltS levels varied as much as 40-fold when *B. licheniformis* cells were grown in the presence of glucose and different nitrogen sources (40, 41). The GltS levels obtained from cells grown in the presence of various carbon sources (15 mM glucose, 45 mM glycerol, 45 mM malate, or 45 mM pyruvate) were not significantly different, suggesting that GltS is not regulated by a carbon catabolite control mechanism. It was previously shown that GltS levels in cells grown in the presence of various nitrogen sources were independent of the growth rate of these cells (41). This was also found to be true when carbon sources were varied (data not shown).

The levels of GltS obtained as a function of culture age from cells grown in the presence of glucose and ammonia showed that GltS activity varied during growth and sporulation (Fig. 3). GltS specific activity dropped dramatically once the culture reached stationary phase, and the values obtained approximately 3 h after the onset of stationary phase represented approximately 15% of the maximum activity. The maximum GltS activity was identical to the activity obtained when cells were grown in continuous culture (40), and those activities measured one generation time before the end of exponential growth reflected the steady-state level of GltS in these cultures.

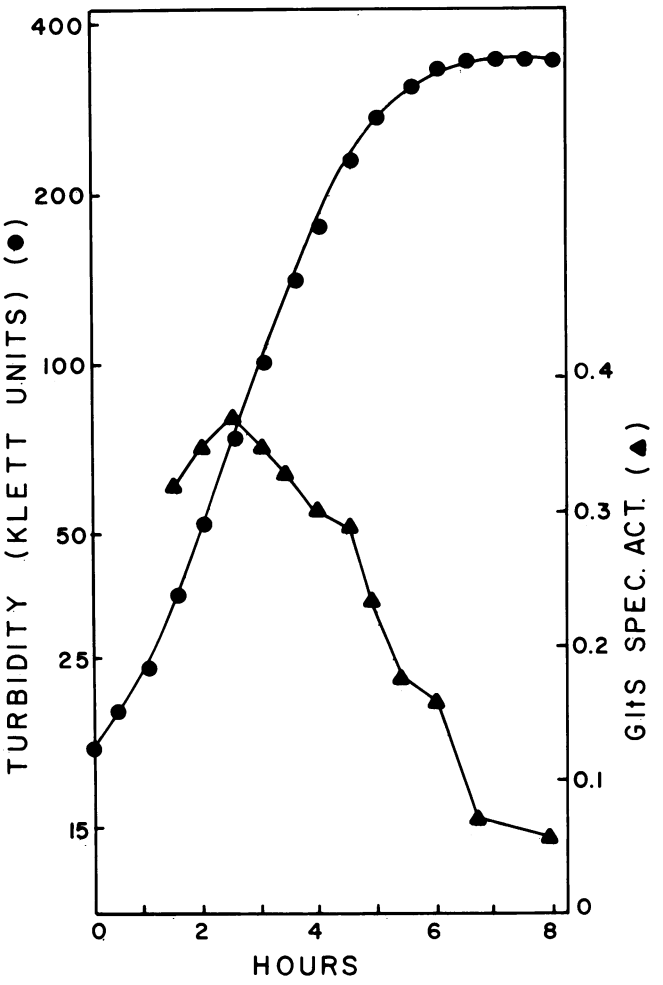

FIG. 3. Specific activity of GltS as a function of culture age. *B. licheniformis* was grown in medium A supplemented with 15 mM glucose and 10 mM $(NH_4)_2SO_4$ as described in the text. Growth was monitored turbidimetrically (○). The cell harvesting conditions used are described in the text. GltS (▲) was assayed under standard conditions.

DISCUSSION

The properties of the GltS purified from *B. licheniformis* are summarized in Table 3. In some respects (e.g., molecular weight of individual subuits, iron-sulfur and flavin composition, and apparent pH optimum), the GltS from *B. licheniformis* appeared to be very similar to the GltSs obtained from other procaryotes (1, 16, 32, 39, 45; Masters and Rowe, Fed. Proc. **38**:724, 1979). However, many differences were found, which indicates that the *B. licheniformis* GltS is unique among the GltSs studied.

Sucrose density gradient sedimentation studies performed under various conditions and dimethyl suberimidate cross-linking studies indicated that the *B. licheniformis* GltS did not aggregate to form higher-molecular-weight species. The amino acid analysis of the *B. licheniformis* GltS showed that it was not similar to the *B. megaterium* and *E. coli* enzymes (Table 2). The *B. licheniformis* enzyme displayed different kinetic properties than the *E. coli* and *B. megaterium* enzymes, and these differences may be reflected in the differences in amino acid composition. Furthermore, all attempts at purifying and stabilizing the *B. licheniformis* GltS by methods utilized for the preparation of the enzymes from *E. coli* and *B. megaterium* were unsuccessful.

We found that the *B. licheniformis* GltS had an absolute requirement for α-ketoglutarate, NADPH, and glutamine. However, glutamine could be replaced by ammonia as the nitrogen donor, and the resulting ammonia-dependent activity was similar to the activities observed for GltS preparations from other organisms (13, 26, 28, 45). This activity, which was usually 2 to 5% of the glutamine-dependent activity of the *B. licheniformis* GltS, was shown to have an apparent pH optimum which was different from that obtained for the *B. licheniformis* GDH. We concluded from this and other studies that the ammonia-dependent activity was probably not due to a contaminating GDH.

The GltS from *B. licheniformis* exhibited typical hyperbolic kinetics for NADPH saturation and was found to have an apparent K_m value of 13 μM for NADPH. However, this enzyme was found to exhibit kinetic characteristics for glutamine and α-ketoglutarate which have not been described for any other GltS studied. Nonhyperbolic biphasic kinetics were obtained for glutamine and α-ketoglutarate

TABLE 3. Properties of *B. licheniformis* GltS

Property	Value
Mol wt	220,000 ± 7,000
$s_{20,w}$	10.2
Mol wt of subunits	54,000 ± 2,000 158,000 ± 5,000
Mol wt of cross-linked prepn	217,000
Quaternary structure	αβ
Iron content per 220,000-dalton dimer	8.1 ± 1.0 atoms
Acid-labile sulfur content per 220,000-dalton dimer	8.1 ± 1.0 atoms
Flavin content per 220,000-dalton dimer	2.1 ± 0.4 moieties
Ratio of flavin adenine dinucleotide to flavin mononucleotide	1.2
UV-visible absorption spectrum (λ_{max})	263, 380, and 450 nm
Fluorescence spectrum (emission)	
280-nm excitation	340 nm
340-nm excitation	530 nm
Apparent pH optimum	
Glutamine dependent	7.8
Ammonia dependent	>8.7
Apparent K_m	
Glutamine	8 and 100 μM
L-Ketoglutarate	6 and 50 μM
NADPH	13 μm

saturation of GltS. Interestingly, the enzyme from *B. megaterium* did not display these kinds of kinetic characteristics and was found to be quite similar to the GltS from other procaryotic sources (16).

Bower and Zalkin (4) showed that the *E. coli* GltS exhibited negative cooperativity upon binding of α-ketoglutarate during the ammonia-dependent reaction. Evidence presented above, as well as the results of fluorescence titration, subunit labeling, and sulfhydryl titration studies (Schreier, Ph.D. thesis), indicates that negative cooperativity may be involved in the binding of α-ketoglutarate and glutamine by the *B. licheniformis* GltS. The addition of glutamate at varying concentrations to the GltS reaction mixture when either glutamine or α-ketoglutarate concentrations were varied did not have an effect on the biphasic nature of the reciprocal plots (data not shown). Many precautions were taken to ensure that the enzyme preparation was not altered during preparation by selective proteolysis (2).

GltSs from other organisms have been shown to be relatively insensitive to regulation by feedback inhibition (1, 32, 39). This was the case for the *B. licheniformis* GltS. Of over 40 compounds tested, 6 amino acids showed a small degree of inhibition of GltS activity, but these would be ineffective at their normal pool levels (6) and probably do not play a significant role in the regulation of the GltS from *B. licheniformis*. It has been shown previously that L-glutamate is not important in regulating the activity of GltSs from other sources, (1, 32). In preliminary experiments we found that L-glutamate acts as both an activator and an inhibitor of the *B. licheniformis* GltS depending on the assay conditions (i.e., glutamine and α-ketoglutarate concentration) and the concentration of glutamate used. This unique aspect of the *B. licheniformis* GltS is being examined further and will be described elsewhere.

The findings that L-methionine-DL-sulfoximine, L-methionine sulfone, and DL-methionine sulfoxide were potent inhibitors of the *B. licheniformis* GltS was significant in light of the work done by other workers who used these compounds to study GltS and glutamine synthetase regulation in the enteric organisms (5). The effect of these compounds on the GltS from a *Bacillus* sp. has not been described previously.

$NADP^+$ was found to be an effective inhibitor of GltS activity. However, an examination of the intracellular pool levels of NADPH and $NADP^+$ in *B. licheniformis* during growth and sporulation indicated that the ratios of $NADP^+$ to NADPH did not approach an inhibitory level, at least under the conditions studied, and the effect of $NADP^+$ on GltS activity is probably not physiologically significant.

GltS levels were examined in extracts of *B. licheniformis* cells grown in the presence of various carbon sources. Our results indicated that GltS levels were not regulated by a carbon catabolite repression mechanism and that the carbon source had little influence on the GltS levels obtained under these cultural conditions. An examination of GltS levels in other organisms grown in the presence of different carbon sources has not been done.

The finding that GltS levels began to drop rapidly at the onset of the stationary phase indicated that GltS may undergo rapid turnover by proteolysis. The decrease in specific activity (a half-life of approximately 1.5 h) was similar to the rate of degradation observed for various enzymes which were not required during sporulation in *Bacillus* spp. (2, 44).

We concluded that *B. licheniformis* contains two enzyme activities for glutamate biosynthesis, GDH and GltS. Both are relatively insensitive to inhibition by known metabolites and are found at high levels in cells growing in a minimal salts medium with glucose and ammonium sulfate as carbon and nitrogen sources (35, 40; this study). The reason(s) for this apparent redundancy in activity is not apparent, but it is interesting that *B. licheniformis* and other *Bacillus* spp. contain unusually large pools of glutamate (6, 40).

ACKNOWLEDGMENTS

This work was supported in part by grants PCM 7924644 and 8121902 from the National Science Foundation.

We thank F. C. Wedler for his generous gift of the purified preparation of *E. coli* glutamine synthetase. The preparation of crude extracts for and the assay of $NADP^+$ and NADPH pools by A. L. Saha is gratefully acknowledged. Suggestions and comments by F. C. Wedler, A. T. Phillips, S. E. Stevens, and T. J. Donohue during the course of this work are appreciated.

LITERATURE CITED

1. **Adachi, K., and I. Suzuki.** 1971. Purification and properties of glutamate synthase from *Thiobacillus thioparus*. J. Bacteriol. **129**:1173–1182.
2. **Bernlohr, R. W., and V. Clark.** 1971. Characterization and regulation of protease synthesis and activity in *Bacillus licheniformis*. J. Bacteriol. **105**:276–283.
3. **Bernlohr, R. W., and B. H. Gray.** 1968. Enzyme inactivation during initiation of sporulation, p. 186–195. *In* L. L. Campbell (ed.), Spores IV. American Society for Microbiology, Washington, D.C.
4. **Bower, S., and H. Zalkin.** 1983. Chemical modification and ligand binding studies with *Escherichia coli* glutamate synthase. Biochemistry **22**:1613–1620.
5. **Brenchley, J. E.** 1973. Effect of methionine sulfoximine and methionine sulfone on glutamate synthesis in *Klebsiella aerogenes*. J. Bacteriol. **114**:666–673.

6. **Clark, V. L., D. E. Peterson, and R. W. Bernlohr.** 1972. Changes in free amino acid production and intracellular amino acid pools of *Bacillus licheniformis* as a function of culture age and growth media. J. Bacteriol. **112**:715–725.
7. **Cook, W. R., J. H. Hoffman, and R. W. Bernlohr.** 1981. Occurrence of an inducible glutaminase in *Bacillus licheniformis*. J. Bacteriol. **148**:365–367.
8. **Davies, G. E., and G. R. Stark.** 1970. Use of dimethyl suberimidate, a cross linking reagent, in studying the subunit structure of oligomeric proteins. Proc. Natl. Acad. Sci. U.S.A. **66**:651–656.
9. **Deuel, T. F., and S. Prusiner.** 1974. Regulation of glutamine synthetase from *Bacillus subtilis* by divalent cations, feedback inhibitors and L-glutamine. J. Biol. Chem. **249**:257–264.
10. **Donohue, T. J., and R. W. Bernlohr.** 1981. Properties of the *Bacillus licheniformis* A5 glutamine synthetase purified from cells grown in the presence of ammonia or nitrate. J. Bacteriol. **147**:589–601.
11. **Donohue, T. J., and R. W. Bernlohr.** 1981. Regulation of the activity of the *Bacillus licheniformis* A5 glutamine synthetase. J. Bacteriol. **148**:174–182.
12. **Edelhoch, H.** 1967. Spectroscopic determination of tryptophan and tyrosine in proteins. Biochemistry **6**:1948–1954.
13. **Geary, L. E., and A. Meister.** 1977. On the methanism of glutamine-dependent reductive amination of α-ketoglutarate catalyzed by glutamate synthase. J. Biol. Chem. **252**:3501–3508.
14. **Habeeb, A. F. S. A.** 1972. Reaction of protein sulfhydryl groups with Ellman's reagent. Methods Enzymol. **25B**:457–464.
15. **Helmerhost, E., and G. B. Stokes.** 1983. Generation of an acid-stable and protein-bound persulfide-like residue in alkali- or sulfhydryl-treated insulin by a mechanism consonant with the β-elimination hypothesis of disulfide bond lysis. Biochemistry **22**:69–75.
16. **Hemmila, I. A., and P. I. Mantsala.** 1978. Purification and properties of glutamate synthase and glutamate dehydrogenase from *Bacillus megaterium*. Biochem. J. **173**:45–52.
17. **Hunt, J. B., P. Z. Smyrniotis, A. Ginsburg, and E. R. Stadtman.** 1975. Metal ion requirement by glutamine synthetase of *E. coli* in catalysis of γ-glutamyl transfer. Arch. Biochem. Biophys. **166**:102–124.
18. **Johnson, C. M., and T. H. Arkley.** 1954. Determination of molybdenum in plant tissue. Anal. Chem. **26**:572–574.
19. **Jorgensen, B. M., and H. N. Rasmussen.** 1979. Recycling analysis of nicotinamide-adenine dinucleotide phosphates (NADP and NADPH). Anal. Biochem. **99**:297–303.
20. **Kalb, V. F., and R. W. Bernlohr.** 1977. A new spectrophotometric assay for protein in cell extracts. Anal. Biochem. **82**:362–371.
21. **King, T. E., and R. O. Morris.** 1967. Determination of acid-labile sulfide and sulfhydryl groups. Methods Enzymol. **10**:634–637.
22. **Klingenberg, M.** 1974. Nicotinamide-adenine dinucleotides: spectrophotometric and fluorometric methods, p. 2045–2059. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analyses, 2nd ed. Academic Press, Inc., New York.
23. **Lovenberg, W., B. B. Buchanan, and J. C. Rabinowitz.** 1963. Studies on the chemical nature of clostridial ferredoxin. J. Biol. Chem. **238**:3899–3913.
24. **Magasanik, B.** 1982. Genetic control of nitrogen assimilation in bacteria. Annu. Rev. Genet. **16**:135–168.
25. **Magasanik, B., and D. M. Rothstein.** 1980. The role of glutamine synthetase in the regulation of bacterial nitrogen metabolism, p. 61–68. *In* J. Mora and R. Palacios (ed.), Glutamine: metabolism, enzymology and regulation. Academic Press, Inc., New York.
26. **Mantsala, P., and H. Zalkin.** 1976. Active subunits of *Escherichia coli* glutamate synthase. J. Bacteriol. **126**:539–541.
27. **Mantsala, P., and H. Zalkin.** 1976. Glutamate synthase: properties of the glutamine-dependent activity. J. Biol. Chem. **251**:3294–3299.
28. **Mantsala, P., and H. Zalkin.** 1976. Properties of apoglutamate synthase and comparison with glutamate dehydrogenase. J. Biol. Chem. **251**:3300–3305.
29. **Martin, R. G., and B. N. Ames.** 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. **236**:1372–1379.
30. **Marzluf, G. A.** 1981. Regulation of nitrogen metabolism and gene expression in fungi. Microbiol. Rev. **45**:437–461.
31. **Masters, D. S., Jr., and A. Meister.** 1982. Inhibition by homocysteine sulfonamide of glutamate synthase purified from *Saccharomyces cerevisiae*. J. Biol. Chem. **257**:8711–8715.
32. **Miller, R. E., and E. R. Stadtman.** 1972. Glutamate synthase from *Escherichia coli*: an iron-sulfide flavoprotein. J. Biol. Chem. **247**:7407–7419.
33. **Mora, J., and R. Palacios (ed.).** 1980. Glutamine: metabolism, enzymology, and regulation. Academic Press, Inc., New York.
34. **Orr, M. D., R. L. Blakeley, and D. Panagou.** 1972. Discontinuous buffer systems for analytical and preparative electrophoresis of enzymes on polyacrylamide gels. Anal. Biochem. **45**:68–85.
35. **Phibbs, P. V., Jr., and R. W. Bernlohr.** 1971. Purification, properties and regulation of glutamic dehydrogenase of *Bacillus licheniformis*. J. Bacteriol. **106**:375–385.
36. **Prusiner, S., and E. R. Stadtman (ed.).** 1973. The enzymes of glutamine metabolism. Academic Press, Inc., New York.
37. **Rao, N. A., S. P. Felton, and F. M. Huennekens.** 1967. Quantitative determination of mitochondrial flavins. Methods Enzymol. **10**:494–495.
38. **Rendina, A. R., and W. H. Orme-Johnson.** 1978. Glutamate synthase: on the kinetic mechanism of the enzyme from *Escherichia coli* W. Biochemistry **17**:5388–5393.
39. **Roon, R. J., H. L. Even, and F. Larimore.** 1974. Glutamate synthase: properties of the reduced nicotinamide adenine dinucleotide-dependent enzyme from *Saccharomyces cerevisiae*. J. Bacteriol. **118**:89–95.
40. **Schreier, H. J., T. M. Smith, and R. W. Bernlohr.** 1982. Regulation of nitrogen catabolic enzymes in *Bacillus* spp. J. Bacteriol. **151**:971–975.
41. **Schreier, H. J., T. M. Smith, T. J. Donohue, and R. W. Bernlohr.** 1981. Regulation of nitrogen metabolism and sporulation in *Bacillus licheniformis*, p. 138–141. *In* H. S. Levinson, A. L. Sonenshein and D. J. Tipper (ed.), Sporulation and germination. American Society for Microbiology, Washington, D.C.
42. **Siegel, W. H., T. Donohue, and R. W. Bernlohr.** 1977. Determination of pools of tricarboxylic acid cycle and related acids in bacteria. Appl. Environ. Microbiol. **34**:512–517.
43. **Sigma Chemical Co.** Bulletin MWS-877. Sigma Chemical Co., St. Louis, Mo.
44. **Switzer, R. L., M. R. Maurizi, J. Y. Wong, and K. L. Flom.** 1979. Selective inactivation and degradation of enzymes in sporulating bacteria, p. 65–79. *In* D. E. Atkinson and C. F. Fox (ed.), Modulation of protein function. Academic Press, Inc., New York.
45. **Trotta, P. P., K. E. B. Platzer, R. H. Haschemeyer, and A. Meister.** 1974. Glutamine-binding subunit of glutamate synthase and partial reactions catalyzed by this glutamine amidotransferase. Proc. Natl. Acad. Sci. U.S.A. **71**:4607–4611.
46. **Tyler, B.** 1978. Regulation of the assimilation of nitrogen compounds. Annu. Rev. Biochem. **47**:1127–1162.
47. **Weber, K., and M. Osborn.** 1975. Proteins and sodium dodecylsulfate: molecular weight determinations on polyacrylamide gels and related procedures, p. 179–223. *In* H. Neurath, R. L. Hill, and C. Boeder (ed.), The proteins, 3rd ed., vol. 3. Academic Press, Inc., New York.
48. **Yagi, K.** 1961. Chemical determination of flavins. Methods Biochem. Anal. **10**:319–356.
49. **Yelton, M. M., and D. C. Yoch.** 1981. Nitrogen metabolism in *Rhodospirillum rubrum*: characterization of glutamate synthase. J. Gen. Microbiol. **123**:335–342.