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Purification and Characterization of Glutamate Synthase from *Azospirillum brasilense*

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Growth conditions for *Azospirillum brasilense* Sp6 were devised for maximal expression of glutamate synthase. The enzyme levels were largely affected by the type and concentration of the nitrogen source. A 10-fold increase in the synthesis of the enzyme was observed at a limiting concentration of ammonia. The enzyme was purified to homogeneity by a procedure which was fairly rapid and allowed a good recovery of enzyme (30%). *Azospirillum* glutamate synthase is a complex iron-sulfur flavoprotein with a stoichiometry of 1 flavin adenine dinucleotide:1 flavin mononucleotide:8 Fe:8 S per protomer with a molecular weight of 185,000. The protomer is composed of two dissimilar subunits with molecular weights of 135,000 and 50,000. Kinetic parameters were determined. K_m values for NADPH, 2-oxoglutarate, and L-glutamine were 6.25, 29, and 450 μM, respectively. The optimum pH was about 7.5. Complete reduction of the enzyme under anaerobic conditions was obtained either by NADPH (in the presence of a regenerating system) or dithionite or by photochemical reduction (in the presence of EDTA and 5-deazariboflavin). No stable long-wavelength intermediates were observed.

Since the pioneering work of Tempest and Meers (27), the enzyme glutamate synthase (EC 1.4.1.13), together with glutamine synthetase, has been considered part of an alternative pathway to glutamate dehydrogenase in nitrogen assimilation. Ammonia is incorporated through glutamine synthetase into glutamine, and then glutamate synthase catalyzes the following reaction: glutamine + 2-oxoglutarate + NADPH→2-glutamate + $NADP^+$, thus providing glutamate for the transaminase reactions and net protein synthesis. This route seems particularly important in nitrogen-fixing microorganisms (18). Glutamate synthase has been found in several bacteria (15, 18) as well as in yeasts and plants (26). The enzyme has been purified to a homogeneous form from relatively few bacteria (9, 16, 24, 28). The bacterial enzyme is an iron-sulfur flavoprotein containing both flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) and is composed of two dissimilar subunits. The molecular weights of the protomer (αβ) are 188,000 for the *Escherichia coli* enzyme (16) and 280,000 for the *Thiobacillus thioparus* enzyme (1). Intermediate values within these limits have been observed for the protomers from other sources (9, 24, 28). Some of the kinetic properties of glutamate synthase have been investigated (23), and a two-site ping-pong (uni-uni, bi-bi) mechanism has been postulated for the reaction, with the flavins and the iron-sulfur clusters functioning as electron carriers between the two sites. However, the electron pathway in the oxidation-reduction reaction and the roles of the single subunits and the various cofactors in such a mechanism remain largely unexplored.

Glutamate synthase activity has also been detected in *Azospirillum* strains (20), and we have reproducibly found such activity during metabolic studies on *Azospirillum brasilense* Sp6, thus confirming earlier findings of Bani et al. (2). *A. brasilense* is a microaerophilic nitrogen-fixing bacterium which associates with the roots of several grasses, including agricultural crops (29). According to several reports, such an association seems to be beneficial for the plant. In addition, preliminary studies on the regulation of nitrogen fixation (*nif*) genes in *Azospirillum* spp. have been published (22), yet the role and regulatory system of glutamate synthase genes are largely unknown. Recently, these genes have been cloned from *E. coli* (6).

Owing to the possible impact of *A. brasilense* on the improvement of agricultural crops, it seemed important to examine more thoroughly the catalytic mechanism of glutamate synthase in this microorganism. In the present paper we report on the growth conditions of the bacterium for the maximal expression of glutamate synthase, the enzyme purification procedure, and some of the physical and chemical properties of the enzyme. A brief comparison is also made of glutamate synthase from *A. brasilense* with the enzyme extracted from other sources.

MATERIALS AND METHODS

All chemicals were reagent grade. DEAE-Trisacryl and Ultrogel AC 22 were obtained from LKB Instruments, Inc. Sepharose-2',5'-ADP was purchased from Pharmacia. Molecular weight standards were from Boehringer GmbH or Pharmacia.

Organism and cultivation. *A. brasilense* Sp6 was originally provided by F. Favilli, University of Florence, Italy, and its general morphological and physiological properties have been described elsewhere (F. Favilli, S. Caroppo, L. Brighigna, and G. Picciuro, Abstr. Commun. Natl. Congr. Soc. Ital. Microbiol., p. 793, 1975.) In growth experiments, several media were tested which differed with regard to the nitrogen source used. Medium A (antibiotic medium) was Difco Laboratories antibiotic medium 3. Medium B (limiting NH_4^+) was a minimal mineral solution (pH 7) containing (in grams per liter) KH_2PO_4 (4.5), $K_2HPO_4 \cdot 3H_2O$ (1.8), $MgSO_4 \cdot 7H_2O$ (0.18), NaCl (0.1), $NaMoO_4 \cdot 2H_2O$ (0.01), $CaCl_2$ (0.01), $FeCl_3 \cdot 6H_2O$ (0.0125), and sodium succinate (5) and supplemented with a limiting amount of ammonia (2.5 mM NH_4Cl). Medium C (excess NH_4^+) was the same as medium B but supplemented with 25 mM NH_4Cl. Medium D

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(N free) was the same as medium B, but without nitrogen source, and was used for growth of the bacteria under nitrogen fixation conditions in Roux bottles. For enzyme preparation, the organism was mass cultured in a Microferm fermentor (New Brunswick Scientific Co., Inc.). Cells were harvested at the end of the exponential phase of growth, i.e., 12 to 15 h after inoculation, except for medium D, in which growth was slower. Sedimented cells could be stored at −20°C.

Enzyme assay. Glutamate synthase and glutamate dehydrogenase activities were assayed spectrophotometrically at 30°C by measuring the initial rates of NADPH or NADH oxidation, respectively, at 340 nm. One unit of activity is the amount of enzyme which oxidizes 1 μmol of NADPH or NADH per min. The standard assay mixture for glutamate synthase contained 50 mM Tris hydrochloride (pH 7.5), 5 mM L-glutamine, 2.5 mM 2-oxoglutarate, 0.2 mM NADPH, and appropriate concentrations of the enzyme in a final volume of 1 ml. When ammonia-dependent glutamate synthase activity was assayed, glutamine was replaced by 100 mM NH_4Cl. The standard assay mixture for glutamate dehydrogenase contained 50 mM Tris hydrochloride (pH 8.1), 100 mM NH_4Cl, 2 mM 2-oxoglutarate, 0.2 mM NADH, and appropriate amounts of the enzyme in a final volume of 3 ml.

Protein determination. Protein content was determined either by the biuret method (8) or by the Lowry method modified as previously described (21). Bovine serum albumin was the reference standard.

Purification procedure. Glutamate synthase was purified from *A. brasilense* Sp6 grown on medium B (limiting NH_4^+). Unless otherwise stated, all procedures were carried out at 4°C.

(i) Step 1: crude cell extract. Washed cells of *A. brasilense* (100 g) were suspended in 10 mM imidazole hydrochloride (pH 7.5) containing 5 mM β-mercaptoethanol, 1 mM EDTA, and 100 mM KCl (buffer A) in a ratio of 1 ml of buffer per 4 g of cells. The cells were ruptured by passage through a French pressure cell (1.42×10^5 kPa) precooled to 4°C. A few micrograms of DNase was added to the extract to decrease the viscosity of the solution. After standing for 30 min, the solution was diluted with 1 volume of buffer A and centrifuged at 100,000 × *g* for 70 min. The supernatant was collected, and the precipitate was washed once with a minimum volume of buffer A and centrifuged again; the supernatants were pooled.

(ii) Step 2: heat treatment. 2-Oxoglutarate was added to the enzyme solution to a final concentration of 2 mM. The solution was then brought to 50°C in a water bath (preheated to 75°C) and maintained at 50°C for 5 min under continuous stirring. After rapid cooling, the solution was centrifuged at 100,000 × *g* for 50 min.

(iii) Step 3: ammonium sulfate precipitation. From the supernatant of the previous step, the 30 to 45% ammonium sulfate fraction was collected and dissolved in a minimum volume of 50 mM imidazole (pH 7.5) containing 5 mM β-mercaptoethanol, 1 mM EDTA, and 1 mM 2-oxoglutarate (buffer B). The solution was dialyzed against four 500-ml changes of buffer B and then clarified by centrifugation.

(iv) Step 4: DEAE chromatography. The enzyme solution was layered on a DEAE-Trisacryl column (37 by 2.5 cm) previously equilibrated with buffer B. The column was washed with a volume of buffer B and then with a volume of buffer B plus 50 mM KCl. Next, a linear gradient from 50 to 150 mM KCl in buffer B was applied to the column. The enzyme peak eluted between 110 and 150 mM KCl, whereas glutamate dehydrogenase came off the column at 80 to 110 mM KCl. The fractions containing glutamate synthase activity were pooled and concentrated under nitrogen in an Amicon Corp. ultrafiltration cell equipped with a YM 30 membrane.

(v) Step 5: gel filtration. The concentrate from the previous step was filtered on an Ultrogel AcA 22 column (73 by 2 cm) previously equilibrated with 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid (PIPES)–KOH (pH 7.5) containing 5 mM β-mercaptoethanol, 1 mM EDTA, and 1 mM 2-oxoglutarate (buffer C). The flow rate was 7 ml/h. The fractions corresponding to the enzyme peak were pooled and concentrated by ultrafiltration as before. Repetition of this step somewhat improves the final purification.

(vi) Step 6: affinity chromatography. The enzyme solution was slowly adjusted to pH 6.5 by addition of 0.2 M HCl. After removal of the denatured protein by centrifugation, the solution was applied to a 2′,5′-ADP–Sepharose column (9 by 1 cm) previously equilibrated with 50 mM PIPES–KOH (pH 6.5) containing 5 mM β-mercaptoethanol, 1 mM EDTA, and 1 mM 2-oxoglutarate (buffer D). The column was washed with one volume of buffer D, which eluted a large peak of contaminant proteins, and then with a volume of buffer C to restore the optimal conditions for glutamate synthase stability. The enzyme was finally eluted with buffer C plus 0.5 M KCl. The fractions were pooled, concentrated by ultrafiltration, and dialyzed against 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–KOH (pH 7.5) containing 5 mM β-mercaptoethanol, 1 mM EDTA, and 1 mM 2-oxoglutarate.

Gel electrophoresis. Sodium dodecyl sulfate (SDS) gel electrophoresis was performed on a slab gel in the vertical LKB apparatus. A linear gradient of acrylamide (4.5 to 22.5%) was used as described by O'Farrell (19) with minor modifications.

Molecular weight determination. The molecular weight of the native enzyme was estimated by gel filtration on an Ultrogel AcA 22 column as reported in the purification procedure. The column was calibrated with proteins of known molecular weight. Molecular weight determination of the enzyme subunits was performed by SDS gel electrophoresis. After staining with Coomassie brilliant blue, gel slabs were scanned with an LKB Ultroscan laser densitometer.

Spectral analysis. Absorbance spectra were determined with a Cary 219 spectrophotometer interfaced with an Apple II Plus computer. Anaerobic experiments were carried out in a special cuvette equipped with side arms and a stopcock as previously described (30). Before each experiment, the entire assembly was alternatively degassed and equilibrated with purified nitrogen for six cycles. Fluorescence spectra were determined with a Jasco FP 550 spectrophotofluorometer.

Determination of flavins, iron, and labile sulfide. Flavins were identified by high-pressure liquid chromatography on a Waters-Jasco apparatus by the method of Light et al. (11). Samples of purified enzyme were boiled for 3 min. After centrifugation, samples of the supernatant were analyzed by reverse-phase chromatography on a C18 μBondapak (Waters Associates, Inc.) analytical column. The eluted peaks were also detected by fluorescence (excitation, 370 nm; emission, 530 nm); the area under the peaks was calculated by the Waters data system. Purified samples of FAD and FMN (11) were used as standards. Iron was determined by the *o*-phenanthroline method (5). Labile sulfide determination was performed by the modified method of Fogo and Popowsky (3).

TABLE 1. Bacterial growth experiments

Medium	Sp act (U/mg)	
	Glutamate synthase	Glutamate dehydrogenase
A (antibiotic medium)	0.008	0.029
B (limiting NH_4^+)	0.085	0.008
C (excess NH_4^+)	0.034	0.018
D (N free)	0.035	0.001

Amino acid analysis. Amino acid analysis was carried out on a Carlo. Erba amino acid analyzer with enzyme samples hydrolyzed in 6 N HCl for 24, 48, and 72 h. Valine and isoleucine were determined at 72 h of hydrolysis; cysteine was determined by dimethyl sulfoxide oxidation (25).

RESULTS

Bacterial growth experiments. To determine the influence of various nitrogen sources on enzyme levels, cells were grown in different media, and the amounts of glutamate synthase and glutamate dehydrogenase were tested. There was an inverse relationship between the specific activities of the two enzymes in media B (limiting NH_4^+ and C (excess NH_4^+), respectively (Table 1). Thus, the level of glutamate synthase was higher at a limiting concentration of ammonia, whereas the reverse was true of glutamate dehydrogenase, which showed the highest activity level in medium A (antibiotic medium) and minimum activity in medium D (N free). These results indicate that glutamate dehydrogenase is scarcely active in the assimilation of ammonia in cells grown under N_2 fixation. Under these conditions, ammonia produced by the nitrogenase is channeled to the glutamine-glutamate synthase pathway. The lower specific activity of glutamate synthase found in medium D (N free) may be related to a higher protein synthesis rate caused by *nif* genes. The maximum activity of glutamate synthase was observed in cells grown on medium B (limiting NH_4^+). Under these conditions, the growth yield was about 2 to 3 g/liter and the doubling time in the logarithmic phase was about 120 min.

Enzyme purification. The enzyme was purified 300-fold with a yield of 25 to 30% (Table 2). The enzyme was homogeneous as judged by gel gradient and SDS gel electrophoresis. It was free of any glutamate dehydrogenase activity and was stable for several months when frozen in liquid nitrogen and stored at −80°C.

Molecular weight and subunit composition. The molecular weight of the native enzyme as determined by gel filtration, was 740,000 ± 10%. SDS gel electrophoresis (Fig. 1) of the purified enzyme showed molecular weight bands of 135,000 and 50,000. Densitometer scanning of the gel showed that the ratio of the areas was 2.72, as expected for equimolar amounts of two dissimilar subunits of these molecular weights.

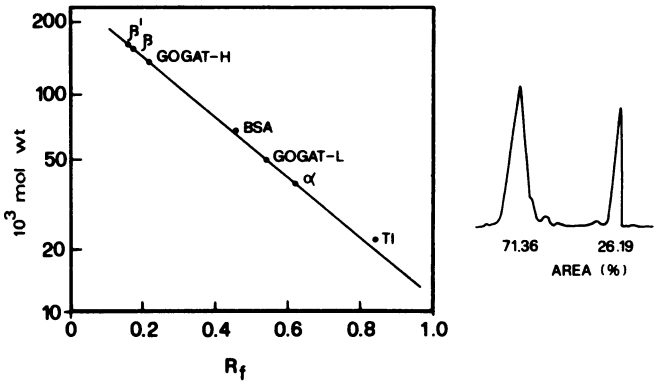

FIG. 1. SDS-polyacrylamide gradient pore gel electrophoresis (4.5 to 22.5%) of glutamate synthase. (A) Calibration curve for estimation of molecular weight: β', β'-RNA polymerase (155,000); β, β-RNA polymerase (145,000); GOGAT-H, glutamate synthase heavy subunit; BSA, bovine serum albumin (66,000); GOGAT-L, glutamate synthase light subunit; α, α-RNA polymerase (39,000); TI, trypsin inhibitor (21,500). (B) Densitometer scanning of the gel.

Flavin, iron, and acid-labile sulfide content. The spectrum of the enzyme (Fig. 2) indicated the presence of flavins and possibly of iron-sulfur clusters in the protein. Flavin content was analyzed by high-pressure liquid chromatography as indicated above. Two peaks corresponding to FMN and FAD were detected. Compared with the standards, the amounts of FAD and FMN found were 1:1 per mole of protomer with a molecular weight of 185,000. The iron and acid-labile sulfur contents were 8.1 iron atoms and 7.9 sulfur atoms per protomer.

Amino acid composition. Table 3 reports the amino acid analysis of glutamate synthase as calculated per mole of protomer with a molecular weight of 185,000; a value of 21 mol of half-cystine was determined. The amino acid composition of glutamate synthase from *A. brasilense* was similar to the previously published analyses of the enzyme from *Bacillus megaterium* (9) and *E. coli* (16). In this respect, these enzymes showed some differences in amino acid composition compared with that of *Bacillus licheniformis* (24).

Spectral properties. The spectrum of the purified enzyme is shown in Fig. 2. Peaks at 443, 376, and 278 nm were detected, with an absorbance ratio of 278/443 = 4.5 to 4.8 for the homogeneous enzyme. The fluorescence spectrum gave emission peaks at 340 and 530 nm (excitation at 250 and 450 nm, respectively); the latter is typical of the flavin chromophore. Flavin fluorescence is largely quenched by the apoprotein; the intensity corresponds to about 10% of

TABLE 2. Glutamate synthase purification scheme

Procedure	Vol (ml)	Activity		Protein (mg/ml)	Sp act	Yield (%)	Purification factor
		U/ml	Total U				
Crude cell extract	164	1.98	325	29.10	0.068	100	
Heat treatment	155	1.97	305	20.70	0.095	94	1.4
$(NH_4)_2SO_4$, 30–45% fraction	28	10.40	291	41.60	0.250	90	3.7
DEAE chromatography	8.3	21.93	182	8.77	2.5	56	36.8
Gel filtration	9.4	13.14	123	1.16	11.3	38	166.4
2',5'-ADP–Sepharose	6.9	13.52	93	0.69	19.5	29	288.2

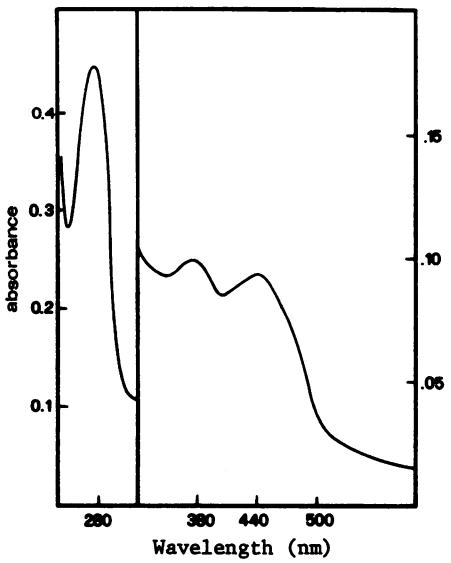

FIG. 2. Absorption spectrum of glutamate synthase (0.388 mg of enzyme per ml in 25 mM HEPES–KOH [pH 7.5] containing 1 mM 2-oxoglutarate, 1 mM EDTA, and 5 mM β-mercaptoethanol).

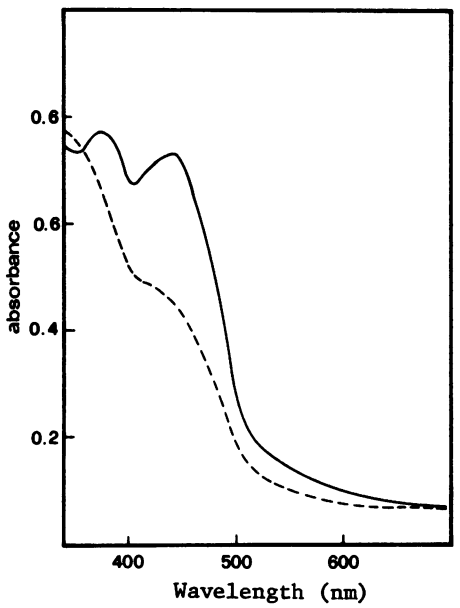

FIG. 3. Reduction of glutamate synthase by an NADPH regeneration system (4.5 μM NADPH, 2 mM glucose-6-phosphate, 10 μg of glucose-6-phosphate dehydrogenase). ——, Oxidized enzyme; – – –, immediately after NADPH addition.

the fluorescence of an equimolar mixture of FMN and FAD. Almost complete reduction of the enzyme was observed when glutamate synthase was anaerobically incubated with NADPH kept reduced by a regenerating system (Fig. 3). Similar spectra were obtained when the enzyme was reduced by dithionite (Fig. 4) or photoreduced by the EDTA-deazariboflavin system (Fig. 5) under anaerobic conditions (13). In the latter case, as well as when NADPH was used, complete reoxidation (98% at 445 nm) was observed on anaerobic addition of 2-oxoglutarate plus glutamine (data not shown); with a fourfold excess of $NADP^+$, only partial reoxidation was obtained (Fig. 5; 70% at 445 nm). In all cases, stable long-wavelength intermediates were not observed during the reduction or oxidation processes.

Kinetic parameters. A pH activity curve gave a broad profile with an optimum of about 7.5. Similarly, the enzyme showed a maximum of stability at a pH between 7.3 and 8.3, with a more rapid decline on the acidic side below pH 6.5. The K_m values of the enzyme for NADPH, 2-oxoglutarate, and L-glutamine were determined under optimal conditions and were 6.25, 29, and 450 μM, respectively. The enzyme was catalytically inactive with NADH (200 μM) as the electron donor. When L-glutamine was replaced by ammonia as the amino-group donor in the assay mixture, the catalytic

TABLE 3. Amino acid composition of glutamate synthase

Amino acid	Mol/mol of protomer (mol wt, 185,000)
Lysine	78
Histidine	41
Arginine	111
Aspartic acid	163
Threonine	93
Serine	73
Glutamic acid	184
Proline	76
Glycine	164
Alanine	159
Half-cystine	21
Valine	131
Methionine	36
Isoleucine	109
Leucine	140
Tyrosine	39
Phenylalanine	55
Tryptophan[a]	ND[a]

[a] ND, Not determined. For the calculations, a tryptophan content of 18 mol/mol of protomer was assumed, as for the *E. coli* enzyme (16).

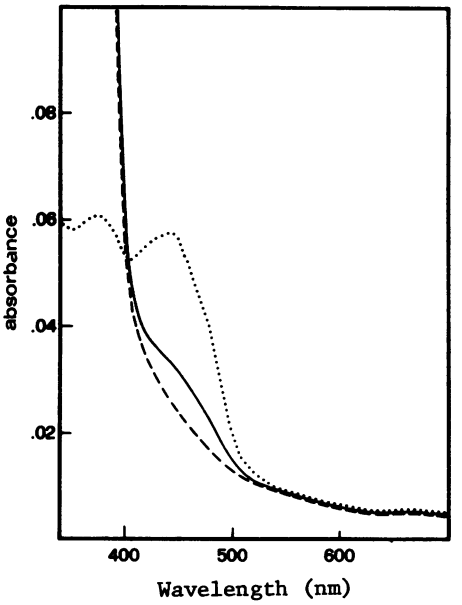

FIG. 4. Dithionite reduction of glutamate synthase. . . . . ., Oxidized enzyme after anaerobiosis was established; ——, immediately after dithionite addition; – – –, 30 min after addition of dithionite.

activity of glutamate synthase was less than 1%. Likewise, when 25 mM L-asparagine was used, enzyme activity decreased to about 0.5% of the original. L-Methionine was a competitive inhibitor of the enzyme with respect to L-glutamine (data not shown), and an apparent K_i value of 1.05 mM for L-methionine was obtained. 6-Diazo-5-oxo-L-norleucine was a potent inhibitor of *A. brasilense* glutamate synthase. Complete loss of glutamine-dependent activity was observed when the enzyme was preincubated for 10 min in the normal assay mixture with 100 μM 6-diazo-5-oxo-L-norleucine; the reaction was started by the addition of glutamine and NADPH. Preliminary studies showed that salts such as KCl and NaCl had no effect on enzyme activity. Among the salts of the chaotropic series, $CaCl_2$ and $NaClO_4$ gave an $I_{0.5}$ of 50 mM, whereas KI and KBr gave $I_{0.5}$ values of 150 and 280 mM, respectively.

DISCUSSION

The isolation and characterization of a glutamate synthase from *A. brasilense* Sp6 emphasize the key role of this enzyme in ammonia assimilation in nitrogen-fixing bacteria. In this respect, the present work showed that, unlike *E. coli* glutamate synthase, the levels of the enzyme in *A. brasilense* are largely affected by the concentration of ammonia salts present in the growth medium. Other nitrogen-fixing microorganisms (18) show similar behavior. The data reported in Table 1 clearly demonstrate that glutamate dehydrogenase activity is repressed in cells grown in limiting concentrations of ammonia, whereas under the same conditions the level of glutamate synthase is increased by one order of magnitude. The reverse is true for cells grown in medium enriched with ammonia salts. Thus, in *A. brasilense*, glutamate synthase represents the major physiological route for nitrogen assimilation under conditions of ammonia starvation.

The present purification procedure was quite rapid and gave a good yield of homogeneous enzyme. A few glutamate synthases have been purified to homogeneity (9, 16, 24, 28). In this respect, the chemical and physical properties of the *A. brasilense* enzyme were similar to those of glutamate synthase from other bacterial sources. For instance, the sizes of the glutamate synthase subunits in *A. brasilense* were almost identical to those of the enzyme from *E. coli* (16). The size of the smaller subunit was practically constant in all of the bacterial enzymes tested (≈50,000). In contrast, the molecular weight of the larger subunit showed significant variation, ranging from 200,000 for the *T. thioparus* enzyme to 135,000 for the enzyme from *E. coli*. The oligomeric structure seemed somewhat different among the various species. Thus, the enzymes from *B. megaterium* (9), *E. coli* (16), and *A. brasilense* show a quaternary structure of the type $\alpha_4\beta_4$, whereas those from *Klebsiella aerogenes* (28), *B. licheniformis* (24), and *T. thioparus* (1) are of the type $\alpha\beta$. In the latter case, the protomer seems to be the active species, whereas with the $\alpha_4\beta_4$ type of glutamate synthase there is no evidence to indicate which of the oligomeric species is involved in catalysis. The *A. brasilense* enzyme, unlike that of *E. coli* W (16), showed catalytic activity when ammonia replaced glutamine as the amino-group donor. Similar NH_3-dependent activity has been detected for glutamate synthase from *E. coli* K-12 (12), *A. aerogenes* (7, 28), and *B. megaterium* (9). The values of the kinetic parameters of the *A. brasilense* enzyme were in the range of those reported for glutamate synthase from other sources. Classical hyperbolic kinetics were observed in all cases (1, 9, 16, 24, 28, 31) except for the enzyme from *B. licheniformis* (24) and the *E. coli* enzyme in the NH_3-dependent reaction (23); in both of these cases biphasic kinetics have been reported. Glutamate synthase from *A. brasilense* is strictly dependent on NADPH as the electron donor, has a typical iron-sulfur flavoprotein spectrum, and contains 8 g-atoms of iron and 8 g-atoms of sulfur per mol of protomer with a molecular weight of 185,000. All of these chemical and physical properties, as well as the presence of FAD and FMN in a 1:1 ratio per protomer as prosthetic groups, are peculiar to glutamate synthases from bacterial sources. Plant and yeast enzymes have higher-molecular-weight subunits and use NADH as an electron donor, and their FAD contents seem somewhat uncertain (14, 26). A class of plant ferredoxin-dependent glutamate synthases has also been reported (10).

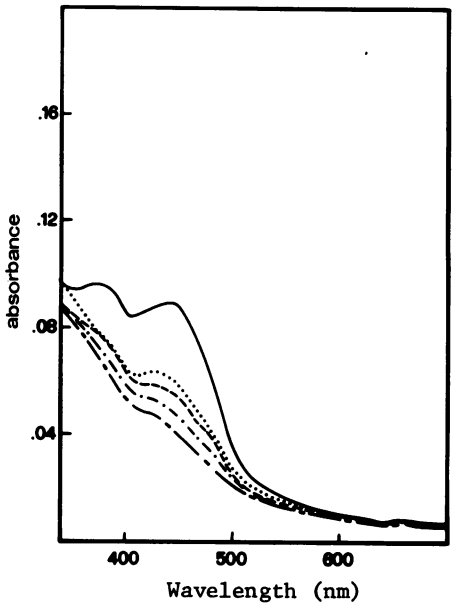

FIG. 5. Photochemical reduction of glutamate synthase in the presence of 8 mM EDTA–0.5 μM 5-deazariboflavin: ——, Oxidized enzyme; – – –, after 60 s of irradiation; - · - ·, 90 s of irradiation; - — -, 210 s of irradiation; · · · · ·, immediately after addition of 50 μl of 0.1 mM $NADP^+$.

Our reduction experiments substantiate the participation of both flavins in electron transfer during catalysis, in contrast to that reported for the *E. coli* enzyme (16). In the latter case, only about 60% reduction of the flavin component was detected on anaerobic reaction of the enzyme with NADPH. Those authors concluded that FAD was the only active flavin in the first part of the catalytic cycle. Alternatively, they postulated the involvement of a flavin semiquinone, and in a later paper (17) the presence of an electron paramagnetic resonance signal (g, 2.00) on anaerobic reduction of the enzyme with NADPH was reported. In our experiments, the extent of spectral bleaching was almost identical with NADPH (in the presence of a regenerating system) or dithionite as a reducing agent, indicating a participation of both of the flavins in the process of reduction by the substrate. Furthermore, we did not observe long-wavelength intermediates with spectral characteristics typical of a flavin semiquinone. The latter was not detected even with photoreduction with EDTA–5-deazariboflavin. These findings may not be in conflict with previous reports (17). In our case, the lack of spectroscopic evidence of a flavin semiquinone could be due to kinetic reasons. However, a detailed study of electron flow in the catalytic mechanism of this enzyme is still lacking, particu-

larly regarding the roles of flavins and iron-sulfur clusters. In this regard, the obtainment of a deflavoenzyme or an apoprotein which can be sequentially reconstituted with the various cofactors of both will allow elucidation of the electron pathway and will give a better insight into the catalytic mechanism. We are currently investigating this possibility.

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