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Purification and Properties of Glutamate Synthase from *Thiobacillus thioparus*

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Glutamate synthase was purified about 250-fold from *Thiobacillus thioparus* and was characterized. The molecular weight was estimated as 280,000 g/mol. The enzyme showed absorption maxima at 280, 380, and 450 nm and was inhibited by Atebrin, suggesting that *T. thioparus* glutamate synthase is a flavoprotein. The enzyme activity was also inhibited by iron chelators and thiol-binding agents. The enzyme was specific for reduced nicotinamide adenine dinucleotide phosphate (NADPH) and α-ketoglutarate, but L-glutamine was partially replaced by ammonia as the amino donor. The K_m values of glutamate synthase for NADPH, α-ketoglutarate, and glutamine were 3.0 μM, 50 μM, and 1.1 mM, respectively. The enzyme had a pH optimum between 7.3 and 7.8. Glutamate synthase from *T. thioparus* was relatively insensitive to feedback inhibition by single amino acids but was sensitive to the combined effects of several amino acids. Enzymes involved in glutamate synthesis in *T. thioparus* were studied. Glutamine synthetase and glutamate synthase, as well as two glutamate dehydrogenases (NADH and NADPH dependent), were present in this organism. The levels of glutamate synthase and glutamate dehydrogenase were similar in *T. thioparus* grown on 0.7 or 7.0 mM ammonium sulfate. The sum of the activities of both glutamate dehydrogenases was only 1/25 of that of glutamate synthase under the assay conditions. It was concluded that the glutamine pathway is important for ammonia assimilation in this autotrophic bacterium.

Microorganisms in a medium with ammonia as the sole source of nitrogen must synthesize amino acids from ammonia to grow. The ammonia assimilation can proceed via either glutamate dehydrogenase or a combination of glutamine synthetase and glutamate synthase (23). The latter pathway has been referred to as the glutamine pathway (24).

In *Klebsiella aerogenes*, it was found that glutamate dehydrogenase operated only under the ammonia excess conditions. Ammonia-limited cultures of *K. aerogenes* repressed glutamate dehydrogenase and produced a high level of glutamate synthase (15). It was suggested that, because of the high affinity of glutamine synthetase for ammonia, this pathway had the capacity to function at levels of free ammonia far below those necessary for the production of glutamate by glutamate dehydrogenase (15, 16). In *Escherichia coli*, however, it was demonstrated that under ammonia-limited conditions glutamate dehydrogenase was progressively induced instead of glutamate synthase, which remained at much the same level during the ammonia starvation (20). It was concluded that *E. coli*, unlike *K. aerogenes*, used glutamate dehydrogenase to incorporate ammonia into glutamate during the ammonia-limited growth (20). In *Salmonella typhimurium*, the glutamate dehydrogenase level did not decrease with limiting ammonia, but mutants of *S. typhimurium* and *K. aerogenes* lacking glutamate synthase activity had similar phenotypes, suggesting the same function for glutamate synthase in both organisms (4).

We have investigated the pathway of ammonia assimilation in *Thiobacillus thioparus*, a chemoautotrophic bacterium, and found a strong glutamate synthase activity in the organism. In this paper we report a partial purification and characterization of a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent glutamate synthase from this organism. A survey of enzymes involved in glutamate synthesis in *T. thioparus* is also presented.

(The results reported here are taken from a thesis presented by K. A. to the University of Manitoba, Winnipeg, Canada, in partial fulfillment of the requirements for the Ph.D. degree, 1976.)

MATERIALS AND METHODS

Organism and growth conditions. An obligate

autotroph, *T. thioparus* ATCC 8158, was obtained from the American Type Culture Collection and grown at 26°C on Starkey no. 2 medium (22), as described previously (12), with a minor modification. The pH of the culture was maintained at 6.5 by the addition of a 15% K_2CO_3 solution by means of a Radiometer pH-stat unit. The yield of wet cells collected with a Sharples centrifuge after 3.5 days of growth was ca. 8 g/20 liters of medium. The cells were stored at −20°C until used for the enzyme preparation.

Enzyme assays. The glutamate synthase activity was determined by following the oxidation of NADPH spectrophotometrically at 340 nm. The reaction mixture contained 0.5 mM α-ketoglutarate, 5 mM L-glutamine, 0.075 mM NADPH, 50 mM tris(hydroxymethyl)aminomethane (Tris)-chloride (pH 7.8), and the enzyme. A unit of the glutamate synthase activity was defined as the amount of enzyme that oxidized 1 μmol of NADPH per min. The activity of glutamine synthetase was determined by the γ-glutamyl-transferase assay described by Shapiro and Stadtman (21). A standard curve was prepared with commercially obtained γ-glutamyl-hydroxamate. One unit of enzyme was defined as the amount required to catalyze the synthesis of 1 μmol of γ-glutamyl-hydroxamate per min. The NADPH-dependent glutamate dehydrogenase was assayed spectrophotometrically by following either oxidation of NADPH or reduction of $NADP^+$ at 340 nm. The standard reaction mixture for the reductive amination procedure contained 160 mM NH_4Cl, 20 mM α-ketoglutarate, 0.15 mM NADPH, 100 mM Tris-chloride (pH 8.5), and the enzyme. For the oxidative deamination assay, the reaction mixture contained 33 mM L-glutamate, 0.33 mM $NADP^+$, and 100 mM glycine-sodium hydroxide (pH 9.5). For the reductive amination assay of the NADH-dependent glutamate dehydrogenase, the standard reaction mixture contained 200 mM NH_4Cl, 20 mM α-ketoglutarate, 0.15 mM NADH, 100 mM Tris-chloride (pH 8.0), and the enzyme. The same reaction mixture as that used for the NADPH-dependent enzyme was used for the oxidative amination assay of the NADH-dependent enzyme, except that $NADP^+$ was replaced by NAD^+. One unit of the L-glutamate dehydrogenases was defined as the amount causing the oxidation of 1 μmol of NADPH or NADH per min with the proper assay mixture.

Purification of glutamate synthase. Routinely, about 30 g (wet weight) of cells was used for the purification of glutamate synthase. Unless otherwise stated, the buffer solution contained 50 mM potasium phosphate (pH 7.5), 5 mM tetrasodium ethylenediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol (DTT) (henceforth referred to as "buffer"). All steps in the purification were performed at 4°C.

Frozen *T. thioparus* cells were suspended in 0.1 M potassium phosphate buffer (pH 7.5) in a ratio of 1 g of cells for each 4 ml of buffer. Cells were disrupted by sonic treatment for 30 min in a 10-kc Raytheon sonic oscillator with the maximum output. The suspension was centrifuged at 48,000 × *g* for 20 min to remove cellular debris. The supernatant fluids were pooled as the crude extract. The crude extract was clarified further by centrifugation at 113,000 × *g* for 60 min. The supernatant liquid was collected and kept frozen at −20°C until needed. It was stable for at least 2 weeks. The supernatant fluid was made 35% saturated in ammonium sulfate. The solution was stirred for 20 min and then centrifuged at 48,000 × *g* for 15 min. The pellet was discarded, and the supernatant solution was adjusted to 50% saturated in ammonium sulfate. After 20 min of stirring, the solution was centrifuged and the supernatant liquid was discarded. The pellet was dissolved in a minimum amount of buffer. The enzyme solution was dialyzed against 100 volumes of buffer for at least 8 h. Insoluble material was removed by centrifugation.

The enzyme solution from the above step was applied to a diethylaminoethyl (DEAE)-cellulose column (2.5 by 40 cm) previously equilibrated with buffer. The protein was eluted with a linear sodium chloride gradient (0 to 0.2 M NaCl made in buffer). Fractions that contained most of the enzyme activity were pooled and dialyzed against buffer. The enzyme solution was made 70% saturated in ammonium sulfate and stirred for 20 min. The precipitate was collected by centrifugation and was suspended in 10 ml of 50% sautrated ammonium sulfate prepared in buffer. After stirring for 30 min the suspension was centrifuged and the supernatant fluid was discarded. The pellet was finally suspended in 5 ml of 35% saturated ammonium sulfate and stirred for 30 min to solubilize the enzyme. The suspension was centrifuged and the pellet was discarded. The supernatant fluid containing the enzyme was dialyzed against 1 liter of buffer. The buffer was changed once after 3 to 4 h, and dialysis was continued overnight.

The enzyme solution from the above step, usually about 10 ml in volume, was applied to a column (2.5 by 92 cm) of Sephadex G-200 previously equilibrated with buffer. The enzyme was eluted with the same buffer, and the highest activity was found in the fraction that eluted when the ratio of elution volume (V_e) to void volume (V_0) was 1.46. The enzyme-containing fractions were pooled and applied to a DEAE-cellulose column (1.5 by 27 cm). The column was eluted in the same manner as for the first DEAE-cellulose column. The fractions with the enzyme activity were pooled, dialyzed overnight against 2 liters of buffer, and used as the purified enzyme.

Protein determination. The protein content of the enzyme solution was determined by the method of Lowry et al. (11), with crystalline bovine serum albumin as the reference protein.

Molecular weight determination. The molecular weight of glutamate synthase was determined by means of gel filtration as described by Andrews (1). A Sephadex G-200 column (2.5 by 92 cm) was equilibrated with a buffer containing 50 mM potassium phosphate (pH 7.5), 5 mM tetrasodium EDTA, and 1 mM DTT. The V_0 was determined with blue dextran 2000 (Pharmacia). Reference proteins used were bo-

vine serum albumin (molecular weight, 68,000), yeast alcohol dehydrogenase (molecular weight, 141,000), bovine liver catalase (molecular weight, 250,000), and milk xanthine oxidase (molecular weight, 290,000) (2). The proteins were dissolved in the same buffer at a ratio of 25 mg/10 ml.

Electrophoresis. Polyacrylamide disc-gel electrophoresis was performed at pH 8.5 by the method of Baker et al. (3). The upper gel was omitted. The gels were polymerized from a mixture containing 5% acrylamide, 0.13% *N,N'*-methylenebisacrylamide (BIS), 25 mM DL-asparagine, 19 mM Tris, 0.07% ammonium persulfate, 0.05% (vol/vol) *N,N,N',N'*-tetramethylethylenediamine (TEMED), and 25% (vol/vol) glycerol. The upper and lower electrode buffers were identical and contained 25 mM DL-asparagine and 19 mM Tris with a final pH of 8.5. Gels were stained with 0.025% Coomassie brilliant blue in 10% acetic acid plus 50% methanol. Destaining solution contained 7.5% acetic acid and 5% methanol. A gel size of 6 by 70 mm was used. The protein solution was prepared in 25% glycerol.

Sodium lauryl (dodecyl) sulfate (SDS)-gel electrophoresis was performed in the presence of 0.1% SDS at pH 7.5 by the procedure of Weber and Osborn (26). The protein solution was boiled for 5 min with 1% SDS and 1% (vol/vol) 2-mercaptoethanol before application. The following proteins were used as the standard polypeptides: hemoglobin (molecular weight, 15,500), yeast alcohol dehydrogenase (molecular weight, 37,000), serum albumin (molecular weight, 68,000), and rabbit muscle phosphorylase *a* (molecular weight, 94,000).

Preparation of affinity gel. Two types of affinity gels were prepared from AH-Sepharose 4B (Pharmacia) with either α-ketoglutarate or L-glutamine as the ligand. In the routine carbodiimide coupling procedure, 2.5 g of freeze-dried AH-Sepharose 4B was laced in a 125-ml beaker and washed five times with 80 ml of 0.5 M NaCl and then five times with 80 ml of distilled water. The coupling mixture contained washed AH-Sepharose 4B (2.5 g, dry weight), 3.7 g of α-ketoglutarate (monopotassium salt) or 1.7 g of L-glutamine, 0.8 g of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, and distilled water to a volume of 40 ml. The mixture was incubated at room temperature for 24 h with gentle propeller stirring. The pH of the solution was kept between 4.5 and 6.0. After the incubation, the gel was thoroughly washed alternately with 0.1 M Tris-chloride buffer (pH 9.0) containing 1.0 M NaCl and with 0.1 M acetate buffer (pH 4.0) containing 1.0 M NaCl to remove noncovalently bound ligands. After a further wash with distilled water, the gel was equilibrated with buffer and packed into a column (1 by 10 cm).

Chemicals. All chemicals and reagents used in this investigation were of analytical grade. Sephadex G-200, AH-Sepharose 4B, and blue dextran 2000 were products of Pharmacia Fine Chemicals. DEAE-cellulose was purchased from Schleicher & Schuell, Inc. DTT, NAD^+, NADH, $NADP^+$, and NADPH were obtained from P-L Biochemicals, Inc. Special enzyme-grade ammonium sulfate was a product of Schwarz/Mann. Acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, and ammonium persulfate were products of J. T. Baker. SDS was supplied by Fisher Scientific Co. All other biochemicals used were obtained from Sigma Chemical Co.

RESULTS

Purification of glutamate synthase. Glutamate synthase was purified more than 240-fold, with an overall yield of 29%, from the *T. thioparus* crude extract (Table 1). The enzyme was eluted after V_0 from a column of Sephadex G-200 (step 6) (Fig. 1), unlike the *A. aerogenes* enzyme, which was eluted in the V_0 (25). In the second DEAE-cellulose chromatography, the elution profile of the enzyme activity was almost coincident with that of protein (absorbancy at 280 nm).

Enzyme purity. The enzyme prepared by the standard procedure described above displayed one major and several minor bands in polyacrylamide gel disc-gel electrophoresis, indicating an incomplete purification. However, no significant competing reaction was found in this enzyme preparation (see "Substrate requirements"), and therefore it was used for the kinetic studies. Attempts were made to purify the enzyme further by affinity gel chromatography. L-Glutamine or α-ketoglutarate was used to prepare the affinity gel as described above. The protein was eluted by several methods including linear gradients of NaCl, KCl, and NH_4Cl and also a 10 mM NADPH solution. The best result was obtained when the α-ketoglutarate affinity gel and a linear gradient of NaCl (0 to 0.3 M prepared in buffer) were used. The enzyme eluted with 0.1 to 0.13 M NaCl by this method displayed one major and one very minor band upon polyacrylamide gel disc-gel electrophoresis. Unfortunately, the enzyme activity decreased considerably during this step, and it was not included in the standard purification procedure.

Absorption spectrum. A yellow component in the enzyme solution prepared by affinity chromatography moved as a single band through the polyacrylamide gel, and after staining it was revealed that the position of the yellow band corresponded to that of the major protein band. The yellow portion of the polyacrylamide gel was cut into small pieces, and the protein was eluted with buffer and assayed for glutamate synthase. It was found that the activity of glutamate synthase was also associated with this yellow protein fraction. The absorption spectrum of glutamate synthase thus purified is shown in Fig. 2. The spectrum of the enzyme exhibits maxima at 280, 380, and 450 nm. The spectral characteristics suggested that

TABLE 1. *Purification of glutamate synthase from T. thioparus*

Fractionation step	Total protein (mg)	Total activity(U)	Sp act (U/mg)	Purification (fold)	Recovery (%)
1. Crude extract	3,120	117	0.0376	1.0	100
2. 113,000 × *g* supernatant	2,360	100	0.0424	1.1	85.5
3. 1st $(NH_4)_2SO_4$, 35–50%	723	77.3	0.114	3.0	66.2
4. 1st DEAE eluate	110	61.0	0.670	18	52.2
5. 2nd $(NH_4)_2SO_4$ fractionation	59.2	58.7	0.990	26	50.2
6. Sephadex G-200 eluate	13.5	39.2	2.90	78	33.5
7. 2nd DEAE eluate	3.6	33.6	9.30	247	28.7

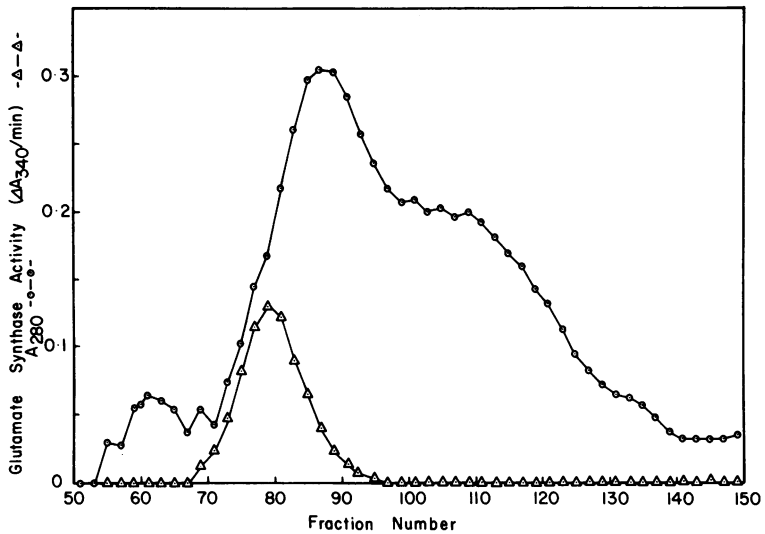

FIG. 1. *Elution profile of glutamate synthase during Sephadex G-200 column chromatography. A sample containing about 60 mg of protein in 12 ml (the second ammonium sulfate fraction) was subjected to chromatography as described in the text. Glutamate synthase activity was measured, using 20 μl of each fraction (2.5 ml). The void volume was at about fraction 54.* A_{340}, *Absorbancy at 340 nm.*

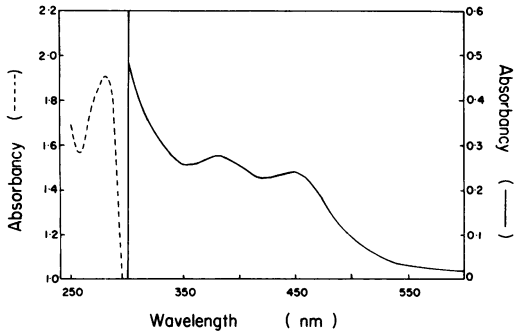

FIG. 2. *Absorption spectrum of glutamate synthase. The enzyme was purified as described in the text and concentrated in a Minicon concentrator (Amicon B15). Protein concentration was 2.5 mg/ml. Light path, 1 cm.*

T. thioparus glutamate synthase is a flavoprotein.

Molecular weight determination. The molecular weight of glutamate synthase was estimated to be 280,000 g/mol by gel filtration on Sephadex G-200. The ratios of V_e to V_0 were 1.45 (xanthine oxidase), 1.46 (glutamate synthase), 1.50 (catalase), 1.63 (alcohol dehydrogenase), and 1.89 (serum albumin).

The SDS-gel electrophoresis of glutamate synthase was performed with the enzyme preparation eluted from the yellow band on polyacrylamide gels. Only two bands appeared after staining: the darker-stained one with a mobility of 0.033 and the lighter one with that of 0.24. Mobility of the standard polypeptides was 0.1 (phosphorylase *a*), 0.31 (serum albumin), 0.46 (alcohol dehydrogenase), and 0.88 (hemoglobin). The approximate molecular weight of the faster-moving subunit of glutamate synthase was estimated as 72,000. The molecular weight of the slower-moving subunit could not be calculated from the standards used but was considerably larger than that of phosphorylase *a*, i.e., 94,000.

Enzyme stability. Stability of glutamate synthase in the absence and the presence of substrates and other compounds was studied before the purification procedures were established. The enzyme obtained from the first ammonium sulfate fractionation (step 3 in purification) prepared without EDTA and DTT was used for this study. The unprotected enzyme was unstable, losing 90% of the activity in 48 h at 4°C. The addition of 5 mM EDTA, 5 mM 2-mercaptoethanol, or 1 mM DTT resulted in enhancement of the enzyme activity and its protection during storage at 4°C, retaining 80 to 90% of the activity after 48 h. At a concentration of 2 mM, NADPH or α-ketoglutarate afforded a relatively slight degree of protection, and L-glutamine, L-glutamate, or $NADP^+$ did not protect the enzyme at all from inactivation. Therefore, the enzyme was purified in the presence of 5 mM EDTA and 1 mM DTT. Storage of purified glutamate synthase at 4°C in the standard buffer resulted in about 40% loss of activity after 10 days. Freezing and storing the purified enzyme at −20°C resulted in a total loss of activity when examined after 10 days. Boiling the enzyme solution for 30 s destroyed the enzyme activity completely.

Substrate requirements. The enzyme had a strict requirement for NADPH as the reduced pyridine nucleotide coenzyme and for α-ketoglutarate as the amino acceptor. NADPH was not replaced with NADH, and α-ketoglutarate could not be replaced with pyruvate or oxalacetate. Although asparagine could not substitute for L-glutamine as the amino donor, NH_4Cl could to a small degree. The ammonia activity with 100 mM NH_4Cl was about 6% of the glutamine activity with 5 mM L-glutamine.

Kinetic parameters. The substrate saturation kinetics for *T. thioparus* glutamate synthase were hyperbolic for all substrates (Fig. 3 through 5). The apparent K_m values for α-ketoglutarate, L-glutamine, and NADPH were 50 μM, 1.1 mM, and 3.0 μM, respectively. The enzyme had a relatively broad pH optimum area occurring between 7.3 and 7.8.

Inhibition by amino acids. The effect of various amino acids on the activity of glutamate synthase was studied (Table 2). Only L-serine inhibited the enzyme activity more than 40% at 10 mM. Four amino acids (L-serine, L-methionine, L-histidine, and L-glutamate) produced more than 50% inhibition at 50 mM.

The effect of a combination of six amino acids on glutamate synthase activity was also studied (Table 3). It was found that the combined effects of the amino acids tested were quite significant, inhibiting 73% of the activity at 7.5 mM each. Although the values of percent inhibition shown in Table 3 agreed with those calculated on the basis of cumulative inhibition (27), there was no evidence for the partial and independent inhibition by individual amino acids, as was the case with glutamine synthetase (27). In fact, single amino acids (e.g., ser-

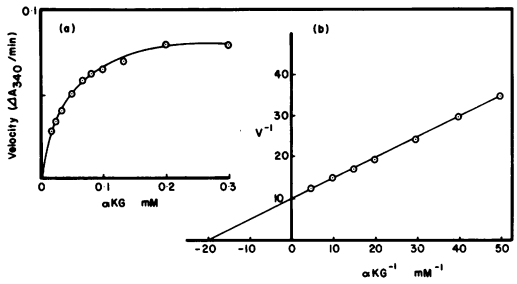

FIG. 3. *Effect of varying α-ketoglutarate concentration on glutamate synthase activity. Standard assay conditions described in the text were used, except that the α-ketoglutarate concentration was varied. (a) Rate-concentration plot; (b) double-reciprocal plot. A_{340}, Absorbancy at 340 nm.*

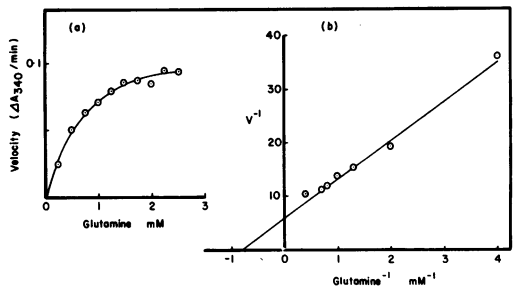

FIG. 4. *Effect of varying L-glutamine concentration on glutamate synthase activity. Standard assay conditions were used, except that the L-glutamine concentration was varied. (a) Rate-concentration plot; (b) double-reciprocal plot. A_{340}, Absorbancy at 340 nm.*

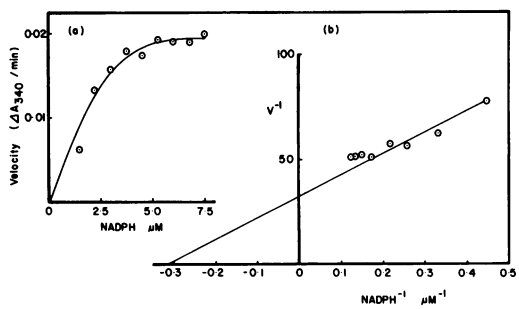

FIG. 5. *Effect of varying NADPH concentration on glutamate synthase activity. Standard assay conditions were used, except that the NADPH concentration was varied. (a) Rate-concentration plot; (b) double-reciprocal plot. A_{340}, Absorbancy at 340 nm.*

TABLE 2. *Inhibition of various amino acids on glutamate synthase activity*[a]

Amino acid	Inhibition (%)	
	10 mM	50 mM
L-Serine	44	76
L-Methionine	36	69
L-Histidine	7	57
L-Glutamate	19	56
Glycine	17	50
DL-Homoserine	8	48
L-Alanine	21	46
L-Lysine	12	39
L-Aspartate	11	38
L-Cysteine	14	37
L-Arginine	7	33
L-Asparagine	5	28
L-Proline	9	24
L-Phenylalanine	5	20
L-Tryptophan	9	19
L-Leucine	0	18
L-Isoleucine	3	7
DL-Threonine	0	7
L-Valine	8	4

[a] Assay mixtures (1 ml) contained 75 μM NADPH, 0.1 mM α-ketoglutarate, 2 mM L-glutamine, 50 mM Tris-chloride (pH 7.8), the indicated amino acid, and 0.45 μg of the purified enzyme. The activity obtained without the amino acid addition was taken as the standard.

TABLE 3. *Effect of a combinatiion of amino acids on glutamate synthase activity*

L-Amino acid[a]						Inhibition (%)
Ser	Met	Ala	Gly	His	Asp	
+	−	−	−	−	−	36
−	+	−	−	−	−	31
−	−	+	−	−	−	15
−	−	−	+	−	−	15
−	−	−	−	+	−	11
−	−	−	−	−	+	8
+	+	−	−	−	−	54
+	+	+	−	−	−	60
+	+	+	+	−	−	64
+	+	+	+	+	−	68
+	+	+	+	+	+	73

[a] Assay conditions were identical to those given in footnote *a* of Table 2. Amino acids were added at a concentration of 7.5 mM each.

ine) could inhibit most of the activity at a high concentration.

Effect of other metabolites. The effect of metabolites other than amino acids on the enzyme activity was studied (Table 4). Of the tricarboxylic acid cycle intermediates tested, only oxalacetate produced more than 50% inhibition at 20 mM. Pyruvate did not produce significant inhibition at the same concentration, but isocitrate inhibited the enzyme by 32%. Among the adenine nucleotides examined, inhibition by adenosine 5'triphosphate (33% at 20 mM) was the highest. $NADP^+$ at 1 mM produced 59% inhibition, whereas NAD^+ had no effect.

Effect of inhibitors. The effect of various inhibitors on glutamate synthase activity is presented in Table 5. The enzyme was inhibited strongly by Atebrin and 2,2'-bipyridyl, suggesting an active catalytic role for flavin and possibly iron in glutamate synthase. Sulfhydryl inhibitors also inhibited the enzyme activity, confirming the importance of thiol groups on this enzyme, which was suggested by the protection by DTT and mercaptoethanol during storage. Azide and cyanide were inhibitory, but only at high concentrations. In a recent report (14) on the *E. coli* enzyme, the alkylation of an essential cysteine was shown to inhibit the glutamine-dependent activity but not the ammonia-dependent activity. The effect of various inhibitors on the ammonia-dependent activity of the *T. thioparus* enzyme was not studied.

Levels of glutamate synthase, glutamate dehydrogenase, and glutamine synthetase. To determine whether glutamate is synthesized in *T. thioparus* by the glutamine pathway, levels of glutamate synthase, glutamate dehydrogenase, and glutamine synthetase were measured. Levels of glutamate synthetase and glutamate dehydrogenase were also tested (Table 6) in *T. thioparus* grown in high-ammonia medium that contained 10 times more ammonium sul-

TABLE 4. *Effect of various metabolites on glutamate synthase activity*[a]

Metabolite	Activity (%)	
	5 mM	20 mM
Cyclic AMP	102	
AMP	102	99
ADP	105	85
ATP	92	67
PEP	93	92
Pyruvate	99	88
Citrate	94	89
cis-Aconitate	95	77
DL-Isocitrate	91	68
Succinate	93	77
Fumarate	88	80
Malate	87	81
Oxalacetate	82	41
$NADP^+$ (1 mM)	41[b]	
NAD^+ (1 mM)	103[b]	

[a] Assay conditions were identical to those given in footnote *a* of Table 2. Abbreviations: AMP, adenosine 5'-monophosphate; ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; PEP, phosphoenolpyruvate.

[b] At a concentration of 1 mM.

TABLE 5. *Effect of inhibitors on glutamate synthase activity*[a]

Inhibitor	Concn (mM)	Inhibition (%)
p-Hydroxymercuribenzoate	1	40
	10	100
	1 (5 min)[b]	100
N-ethylmaleimide	5	14
	10	83
	5 (5 min)[b]	29
Iodoacetic acid	5	0
	25	27
Sodium arsenite	20	10
	50	100
Atebrin	0.2	17
	0.5	77
	1	100
2,2'-Bipyridyl	1	0
	3	100
o-Phenanthroline	5	11
	10	37
NaN_3	20	11
	50	100
KCN	5	12
	20	89

[a] The standard reaction mixture was used except that the indicated compound was added.

[b] The enzyme was preincubated for 5 min with the inhibitor before the initiation of reaction.

fate than the standard medium. Two distinct glutamate dehydrogenases, one specific for NADH and another for NADPH, were found in *T. thioparus* which could be partially separated by means of ammonium sulfate fractionation (Table 7).

In *T. thioparus* grown under the standard conditions, it was found that the combined activity of both glutamate dehydrogenases was only 4% that of glutamate synthase under the assay conditions used (Table 6). The levels of these enzymes did not change appreciably when *T. thioparus* was grown under the high-ammonia conditions. It was found that the activity of glutamine synthetase in *T. thioparus* was of the same order of magnitude as that in *E. coli* (16). The level of enzymes in the organism grown at an ammonia concentration below that of the standard medium was not investigated.

NADP-dependent glutamate dehydrogenase was purified further from fraction 2 of Table 7 by dialysis against 50% saturated ammonium sulfate, removal of the precipitate, dialysis of the supernatant against 58% saturated ammonium sulfate, and finally centrifugation to collect the enzyme. The enzyme was purified 38-fold over the crude extract. The substrate saturation kinetics were all hyperbolic (data not presented). The apparent K_m values for α-ketoglutarate, ammonia, and NADPH were 370 μM, 23 mM, and 80 μM, respectively, for reductive amination. The values for glutamate and $NADP^+$ were 870 μM and 50 μM, respectively, for oxidative deamination.

NAD-dependent glutamate dehydrogenase

TABLE 6. *Levels of glutamate synthase, glutamate dehydrogenase, and glutamine synthetase in T. thioparus*

Enzyme	Coenzyme	Sp act[a] (U/mg)	
		Standard conditions	High ammonia[b]
Glutamate synthase	NADPH	0.0626	0.0593
	NADH	0.0000	NT[c]
Glutamate dehydrogenase	NADPH	0.0018	0.0016
	NADH	0.0009	0.0008
Glutamine synthetase		0.115	NT

[a] Assay conditions were those described in the text. The supernatant from step 2 in the purification of glutamate synthase (113,000 × *g* supernatant) was used for the assay of these enzymes.

[b] A 10 times higher concentration of $(NH_4)_2SO_4$ (7.6 mM) was added to the medium.

[c] NT, Not tested.

TABLE 7. *Separation of two species of glutamate dehydrogenase*[a]

Ammonium sulfate fraction (% saturation)	Glutamate dehydrogenase activity (total units)	
	NADPH activity	NADH activity
1. 40–50	0.052	0.152
2. 50–60	0.222	0.072
3. 60–70	0.005	0.000

[a] *T. thioparus* cells (4 g, wet weight) were sonically oscillated, centrifuged, and ultracentrifuged as described in Table 1. The supernatant was applied to a DEAE-cellulose column (2.5 by 40 cm). After a wash with 50 mM potassium phosphate (pH 7.5), the glutamate dehydrogenases were eluted with 0.15 M potassium phosphate at pH 7.5. The DEAE eluate containing 50 mg of protein was subjected to fractionation with ammonium sulfate. Glutamate dehydrogenase activity was assayed as described in the text and is expressed as total units in each fraction.

was not studied extensively, but demonstrated only the reductive amination activity and not the oxidative deamination reaction.

DISCUSSION

The results reported in this paper establish the presence of glutamate synthase in a chemoautotrophic bacterium, *T. thioparus*. Although the enzyme preparation purified by standard procedures given in Table 1 still contained several minor contaminating protein components, as revealed by polyacrylamide gel electrophoresis, it was possible after affinity gel chromatography and polyacrylamide gel electrophoresis to obtain the spectrum of pure glutamate synthase (Fig. 2). Glutamate synthase purified from either *E. coli* (16) or *K. aerogenes* (25) is an iron-sulfur flavoprotein containing both riboflavin 5'-phosphate and flavin adenine dinucleotide. The spectrum of the *T. thioparus* enzyme and the inhibition of activity by Atebrin (Table 5) suggest that this enzyme is also a flavoprotein and that flavin is involved in its catalytic action. The inhibition studies (Table 5) suggest that the *T. thioparus* enzyme may also be an iron-sulfur flavoprotein, although more direct evidence is required for a definite conclusion. The molecular weight of 280,000 was much smaller than that for the *E. coli* enzyme in its polymeric state (800,000) (16) and was slightly higher than that for the *K. aerogenes* enzyme monomer (227,000) (25). Because of an inherent error in the method used and a possible effect of protein association, the value has to be considered tentative. SDS-polyacrylamide gel electrophoresis of glutamate synthase from *E. coli* and *K. aerogenes* revealed that the enzyme consisted of two dissimilar subunits with molecular weights of 135,000 and 53,000 for the *E. coli* enzyme (16) and 175,000 and 51,500 for the *K. aerogenes* enzyme (25). A preliminary study suggested that the *T. thioparus* enzyme was also composed of dissimilar subunits, with the smaller subunit having a molecular weight of 72,000. Although the molecular weight of the larger subunit could not be determined in the present study, it would be around 200,000 if a molecular weight of 280,000 were assumed for a monomer consisting of one of each subunit. Unfortunately, the small amount of cells available of this chemoautotrophic bacterium could not supply a sufficient quantity of enzyme for its detailed physical or chemical study. The molecular weights of the subunits, therefore, should be considered quite tentative. Recently the *E. coli* enzyme was dissociated into two active subunits, the smaller subunit catalyzing the ammonia-dependent synthesis of glutamate and the larger one exhibiting glutaminase activity (13).

The optimal pH range for *T. thioparus* glutamate synthase activity (pH 7.3 to 7.8) is similar to that for the enzymes from *Saccharomyces cerevisiae* (pH 7.1 to 7.7), *E. coli* (pH 7.6), and *K. aerogenes* (pH 7.8) (18, 16, 25).

The K_m value of *T. thioparus* glutamate synthase for NADPH was very low (3.0 μM), making the accurate determination of reaction velocity difficult in experiments of Fig. 5. This value, however, is of an order of magnitude similar to the K_m values 2.6 μM, 7.7 μM, and 12 μM for the enzymes from *S. cerevisiae* (18), *E. coli* (16), and *K. aerogenes* (25), respectively. The K_m values of the *T. thioparus* enzyme for α-ketoglutarate (50 μM) and L-glutamine (1.1 mM) are compared with those reported for the yeast enzyme (0.14 and 1.0 mM [18]), the *E. coli* enzyme (7.3 μM and 0.25 mM [16]), and the *K. aerogenes* enzyme (0.3 and 0.3 mM [25]).

T. thioparus glutamate synthase was highly specific for NADPH as the coenzyme and α-ketoglutarate as the amino acceptor. However, it was found that NH_4Cl could substitute for L-glutamine to a small degree. The activity obtained with 100 mM NH_4Cl was about 6% of the glutamine activity (with 5 mM L-glutamine). The ratio of the activities was similar to that reported for the purified *K. aerogenes* enzyme, which was apparently free from glutamate dehydrogenase (25). In the *K. aerogenes* system, it was concluded that the ammonia activity was a partial reaction of the overall reaction catalyzed by glutamate synthase. A recent paper (13) on the *E. coli* system identified the smaller subunit of glutamate synthase as the site of ammonia-dependent glutamate synthase activity.

Of 19 amino acids tested (Table 2), only 4 inhibited the *T. thioparus* enzyme more than 50% at 50 mM, whereas 10 produced more than 50% inhibition in the *E. coli* enzyme at 50 mM (16). At 50 mM, L-aspartate and L-methionine inhibited the *E. coli* enzyme more than 90%, whereas only L-serine inhibited the *T. thioparus* enzyme more than 70%. Thus, the *E. coli* enzyme seems to be more susceptible to feedback inhibition by amino acids than the *T. thioparus* enzyme. With the latter enzyme, however, the inhibition by six amino acids tested showed that a combined effect of these amino acids could exert a strong feedback control on the enzyme. The mechanism of this combined inhibition requires further study and may not be identical to the well-described cumulative inhibition of glutamine synthetase system (27).

Among other metabolites tested (Table 4), $NADP^+$ was the strongest inhibitor of the en-

zyme, possibly acting as a product inhibitor of the reaction. Oxalacetate as an analogue of α-ketoglutarate was also inhibitory.

An obligate autotroph, *T. thioparus*, seems to have two distinct glutamate dehydrogenases (Tables 6 and 7). A similar case has been reported for facultative autotrophs *T. novellus* (9) and *Hydrogenomonas* H16 (7) and for a number of fungi (5, 6, 8, 10, 19). It has been suggested that the NADPH-dependent glutamate dehydrogenase operates biosynthetically, whereas the function of NADH-dependent glutamate dehydrogenase is the catabolism of glutamate (19). The K_m value for ammonia of the NADPH-dependent enzyme of *T. thioparus*, however, is very high (23 mM), and the enzyme is not expected to operate biosynthetically unless there is an ample supply of ammonia. Although the K_m of NADH-dependent glutamate dehydrogenase was not accurately determined, it was estimated to be close to that of the NADPH-dependent enzyme, and its near maximum activity was obtained with an NH_4Cl concentration of 200 mM.

The level of glutamate synthase detected in *T. thioparus* was approximately 25-fold higher than the sum of both activities of the two glutamate dehydrogenases measured in the direction of glutamate synthesis (Table 6). Since a high level of glutamine synthetase activity was also detected, it was concluded that *T. thioparus* possessed the glutamine pathway to synthesize glutamate. The activity of glutamate synthase was not detected in cells of *K. aerogenes* grown under the ammonia excess conditions (15). However, *T. thioparus* grown in the high-ammonia medium produced approximately the same levels of glutamate synthase and glutamate dehydrogenases as in the standard medium. This apparent lack of response to "high ammonia" could have been due to the presence of sufficiently high ammonia for the autotrophic growth (0.4 g of wet cells per liter) in the standard medium (0.1 g of ammonium sulfate per liter). The effect of the ammonia-limited conditions on the enzyme composition should be studied in a continuous chemostat culture of *T. thioparus*. In *E. coli*, levels of glutamate synthase and glutamate dehydrogenase were relatively unaffected by the concentration of ammonia in batch cultures (16), but the ammonia-limited chemostat culture of *E. coli* progressively produced increasing levels of glutamate dehydrogenase to incorporate ammonia (20).

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