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Nitrogen and Ammonia Assimilation in the Cyanobacteria: Purification of Glutamine Synthetase from *Anabaena* sp. Strain CA

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Glutamine synthetase was purified from the cyanobacterium *Anabaena* sp. strain CA, a newly isolated marine organism. This organism grows rapidly under nitrogen-fixing conditions and therefore is ideally suited for studies concerning cyanobacterial nitrogen metabolism. Studies were conducted to optimize the production of glutamine synthetase by *Anabaena* CA. The highest specific activities were obtained from cells grown in the presence of atmospheric N_2 or KNO_3 (13 mM); when NH_4Cl was used as the nitrogen source, the specific activity was reduced by approximately 40%. Furthermore, through the use of a whole-cell γ-glutamylhydroxamate transferase assay, it was found that the maximum number of enzyme units is obtained in the late logarithmic stage of growth. Glutamine synthetase purification requires only three steps and results in a preparation that is electrophoretically homogeneous. The transferase specific activity (units per milligram of protein) of the purified enzyme is 78, whereas the biosynthetic specific activity is 2.2. The molecular weight of the native protein was found to be approximately 590,000, and the subunit molecular weight was determined to be about 50,000. Thus, this cyanobacterial enzyme closely resembles the enzyme obtained from other procaryotic sources, at least with regard to size. The purification of glutamine synthetase from *Anabaena* CA should stimulate a more detailed study of this enzyme and its role in cyanobacterial nitrogen metabolism.

In recent years there has been an upsurge of interest in nitrogen fixation catalyzed by the cyanobacteria. However, little is known about the metabolic fate of the nitrogen once it is fixed. Many organisms are known to incorporate ammonia by the enzyme glutamate dehydrogenase, yet this enzyme has been found to be absent or present in low amounts in the cyanobacteria (5, 17). Recent work has shown that under conditions of ammonia limitation most procaryotes use a pathway consisting of glutamine synthetase (L-glutamate:ammonia ligase [adenosine 5'-diphosphate forming], EC 6.3.1.2.) and glutamate synthase (L-glutamine:2-oxoglutarate aminotransferase, EC 1.4.7.1.) to assimilate ammonia (13). In the cyanobacteria, this pathway has been shown to be the major ammonia assimilatory route under nitrogen-fixing conditions (27, 32).

The first enzyme of this pathway, glutamine synthetase, has been rigorously studied in many procaryotes, and these results have been extensively reviewed (6, 20, 22-24). However, little is known concerning the regulation and molecular characteristics of the enzymes involved in nitrogen and ammonia assimilation in the cyanobacteria, organisms that increasingly are being recognized for their importance in global nitrogen fixation (4). The work described in this report represents the first preparation of electrophoretically homogenous glutamine synthetase from a cyanobacterium. Data are presented indicating that the cyanobacterial enzyme, at least structurally, closely resembles the enzyme obtained from other procaryotic sources.

MATERIALS AND METHODS

Reagents. All biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo. All common compounds were reagent-grade quality. Molar stock solutions of imidazole were clarified by charcoal filtration before use. Bio-Gel A-1.5m, 200 to 400 mesh, was obtained from Bio-Rad Laboratories, Richmond, Calif.

Organism and growth. *Anabaena* sp. strain CA is a recently isolated, filamentous, marine, nitrogen-fixing cyanobacterium and was used exclusively in this investigation. This organism is attractive

for nitrogen-assimilatory studies due to its high growth rate on molecular nitrogen (G. Stacey, C. Van Baalen, and F. R. Tabita, Arch. Microbiol., in press). The characteristics and growth requirements of this organism have been recently described (Stacey et al., in press). The organism was grown at 39°C and bubbled with 1% CO_2 in air in minimal salts medium ASP-2 (19, 29). Cells were harvested and the cell paste obtained was used immediately.

Enzyme assays. One unit of enzyme activity is defined as that amount of enzyme needed to form 1 μmol of product per min at 37°C under optimal assay conditions. The transferase activity of glutamine synthetase was determined at pH 7.0 by measuring the amount of γ-glutamylhydroxamate formed, by the method of Shapiro and Stadtman (21). Whole-cell glutamine synthetase activity was determined by the above assay after toluene treatment of cells. In this treatment, a 0.5-ml cell suspension is mixed with 0.25 ml of toluene and incubated for 10 min at 4°C. The cells are then centrifuged, the toluene layer is removed, and the cell pellet is suspended in a 0.5-ml volume of 50 mM imidazole-hydrochloride buffer (pH 7.0) containing 5 mM $MgCl_2 \cdot 6H_2O$ and 10 mM glutamate (buffer A). Such assays were shown to reflect glutamine synthetase activity accurately since, for a given portion of cells, whole-cell determinations gave the same total number of glutamine synthetase units when compared to cell-free enzymatic activity. In all cases, it was shown that the measurements were linear with cell concentration and time of assay. The biosynthetic activity of glutamine synthetase was assayed by a modification of the procedure of Woolfolk et al. (33). The assay mixture was as follows, in a final volume of 0.2 ml: adenosine 5′-triphosphate, 7.5 μmol; $MgCl_2 \cdot 6H_2O$, 50 μmol; monosodium glutamate, 100 μmol; *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer-NaOH (pH 7.0), 50 μmol.

Protein was determined by the method of Lowry et al. (11), with crystalline bovine serum albumin as the protein standard. In the case of purified-enzyme solutions, protein concentrations were estimated by the absorption of ultraviolet light at 280 and 260 nm as described by Layne et al. (9).

Electrophoresis. Discontinuous gel electrophoresis of the purified protein was carried out by using a system containing triethanolamine and *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid (TEA-TES system) as described by Orr et al. (15). Gels were stained with Coomassie brilliant blue as previously described (26). Sodium dodecyl sulfate-discontinuous gel electrophoresis and staining of sodium dodecyl sulfate gels were performed by standard procedures (30).

Purification of glutamine synthetase. Freshly harvested cells (54 g, wet weight) were suspended in buffer A and passed twice through a chilled French pressure cell at 20,000 lb/in^2. Cell debris was removed by centrifugation at 16,300 × *g* for 10 min. The resulting supernatant fluid was clarified by high-speed centrifugation at 105,000 × *g* for 1 h. The supernatant fluid from this step represents the crude extract obtained under all growth conditions.

The high-speed supernatant fluid was made 25% in $(NH_4)_2SO_4$, at 4°C, by adding solid $(NH_4)_2SO_4$ slowly with stirring. The suspension was allowed to sit for 30 min at 4°C and then was centrifuged at 16,300 × *g* for 30 min. The resulting supernatant fluid was then made 50% in $(NH_4)_2SO_4$ in a similar fashion. After sitting for 30 min, the mixture was centrifuged as described above. The supernatant fluid obtained was then made 60% in $(NH_4)_2SO_4$ by the addition of solid $(NH_4)_2SO_4$ with stirring. After 30 min, the glutamine synthetase-containing protein pellet was obtained by centrifugation at 27,000 × *g* for 30 min. This pellet was suspended in a minimum volume of buffer and dialyzed overnight. The dialyzed extract was applied to a 100-ml column of diethylaminoethyl-cellulose that had been equilibrated with several column volumes of the above buffer. After the extract was applied, the column was washed with buffer until the absorbance at 280 nm of the eluate approached 0.05. The column was then eluted with buffer containing 0.1 M NaCl until the absorbance at 280 nm again approached 0.05. A 500-ml gradient consisting of 250 ml of 0.1 M NaCl-containing buffer and 250 ml of buffer containing 0.4 M NaCl was then applied. The enzyme eluted between 0.2 and 0.3 M NaCl. High-specific-activity fractions were pooled and concentrated with an Amicon ultrafiltration cell (Amicon Corp., Lexington, Mass.) equipped with an XM-50 filter. The concentrated protein solution was then subjected to dialysis overnight against a 200-fold excess of buffer.

As a final step in the purification, the concentrated protein solution (12.4 ml) obtained after diethylaminoethyl-cellulose fractionation was layered in 2.0-ml portions onto discontinuous 0.2 to 0.8 M sucrose gradients prepared in the usual buffer. For each gradient, 8 ml of 0.8 M sucrose was pipetted to the bottom of the tube, followed in succession by 8 ml each of 0.6, 0.4, and 0.2 M sucrose. All gradients including the 2-ml sample were prepared to a total volume of 34 ml and centrifuged at 80,000 × *g* for 36 h in a Beckman SW25.1 rotor (Beckman Instruments, Inc., Fullerton, Calif.). When centrifugation was completed, 1-ml fractions were collected. The fractions containing the highest specific activity were then pooled and tested for homogeneity by discontinuous gel electrophoresis.

RESULTS

Glutamine synthetase and growth. In many procaryotes, the expression of glutamine synthetase has been shown to depend directly on the nitrogen content of the growth milieu (20, 22, 24). Not surprisingly, *Anabaena* CA showed the highest level of activity when cultured under nitrogen-fixing conditions, reaching a specific (transferase) activity of 2.5 U/mg of protein. Cells grown with saturating amounts of NH_4Cl (13 mM) yielded a specific activity of 1.6 U/mg of protein, or about 40% of the value obtained under nitrogen-fixing conditions. Interestingly, when cells were grown with saturating levels of nitrate (13 mM), the specific

activity obtained was precisely the same as for cells grown with molecular nitrogen. Such results, with three common cyanobacterial nitrogen sources, were completely reproducible using early- or mid-exponential-phase cells.

Using toluene-treated whole cells, we followed the activity of glutamine synthetase throughout the growth phase under nitrogen-fixing conditions. The synthesis of glutamine synthetase paralleled growth of *Anabaena* CA closely (Fig. 1). Thus, the greatest number of enzyme units was found in late-logarithmic-phase cells. Yet cells at this stage of growth (i.e., 48 h) possessed lower levels of glutamine synthetase (0.17 U/ml per optical density unit) than early-log-phase cells (0.26 U/ml per optical density unit at 11 h). However, to obtain maximum levels of total enzyme units for subsequent enzyme purification, late-logarithmic-phase cells were used. Such an experiment also illustrates the care one must exercise in comparing glutamine synthetase levels when different nitrogen sources are used. In all cases in this investigation, when cells were compared under different growth conditions, precautions were taken to ensure that maximum levels of enzyme activity were measured.

Purification of glutamine synthetase. Two major proteins were found to contaminate our first preparations of glutamine synthetase. These contaminants were shown to be ribulose 1,5-bisphosphate (Rbu-P_2) carboxylase and the phycobiliprotein phycocyanin. Previously, it was shown that Rbu-P_2 carboxylase precipitated from solution upon the addition of $(NH_4)_2SO_4$ to 50% saturation (25). By this method, all of the contaminating Rbu-P_2 carboxylase activity was removed, leaving glutamine synthetase in the supernatant fraction. Subsequently, the glutamine synthetase was precipitated by the further addition of $(NH_4)_2SO_4$ to 60% saturation. The second step of the purification, chromatography on diethylaminoethyl-cellulose, was found to remove most of the remaining phycocyanin and the bulk of the other soluble protein contaminants (Fig. 2). Isopycnic centrifugation on 0.2 to 0.8 M sucrose gradients was used as a final purification step (Fig. 3).

The purification of glutamine synthetase from *Anabaena* sp. CA is summarized in Table 1. This procedure requires only three steps and results in electrophoretically homogeneous enzyme (Fig. 4). The homogeneous enzyme, as measured by transferase activity, was obtained in approximately 17% yield with a specific activity of 78 U/mg of protein. The total purification was 57-fold, indicating that glutamine synthetase comprised approximately 2% of the total soluble protein under the growth condi-

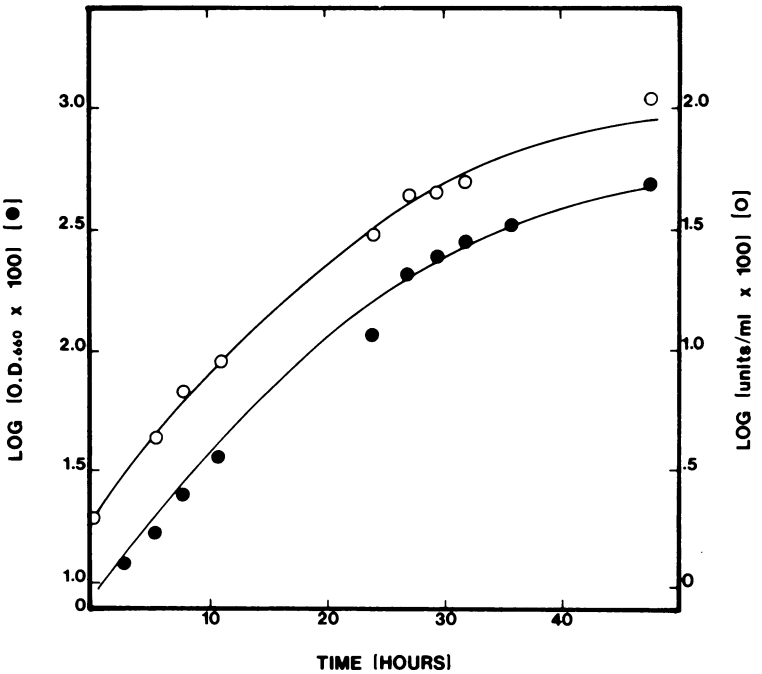

FIG. 1. *Activity of glutamine synthetase during the growth of Anabaena sp. CA under nitrogen-fixing conditions. Enzyme activity was determined by the whole-cell γ-glutamylhydroxamate transferase assay.*

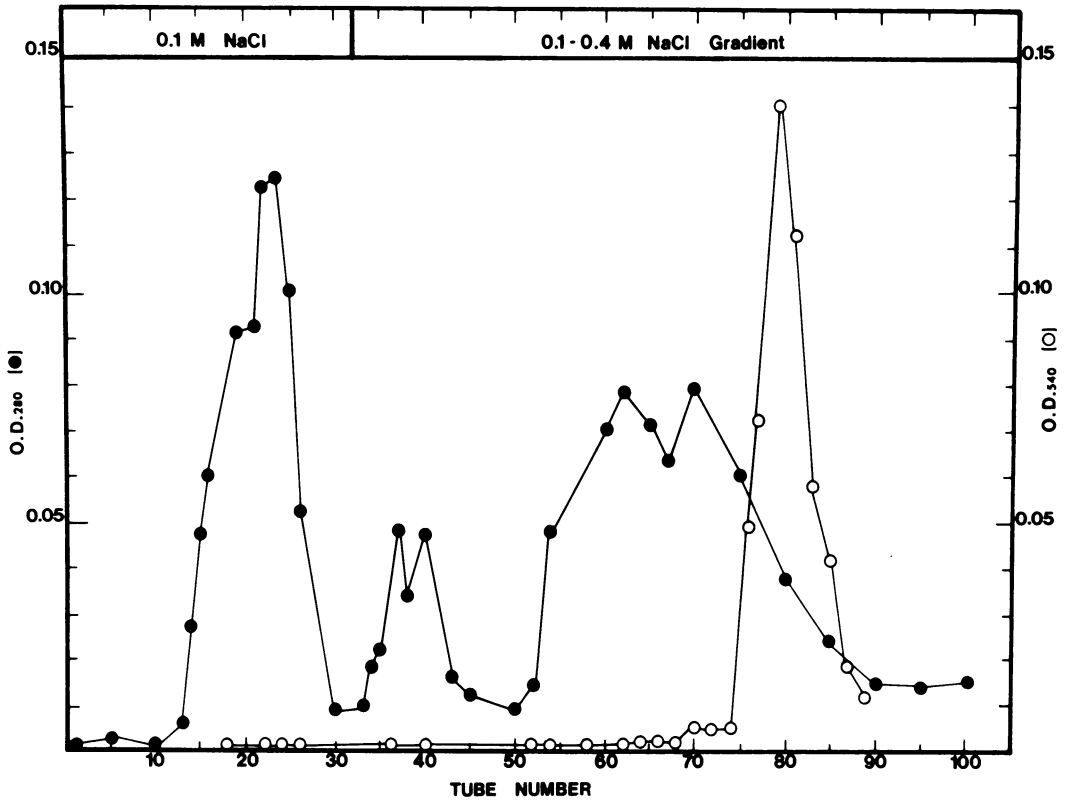

FIG. 2. *Diethylaminoethyl-cellulose chromatography profile of a cell extract after $(NH_4)_2SO_4$ fractionation. The procedures used are indicated in text. Enzymatic activity is expressed in terms of the optical density at 540 nm (OD_{540}) after 20 min in the transferase assay.*

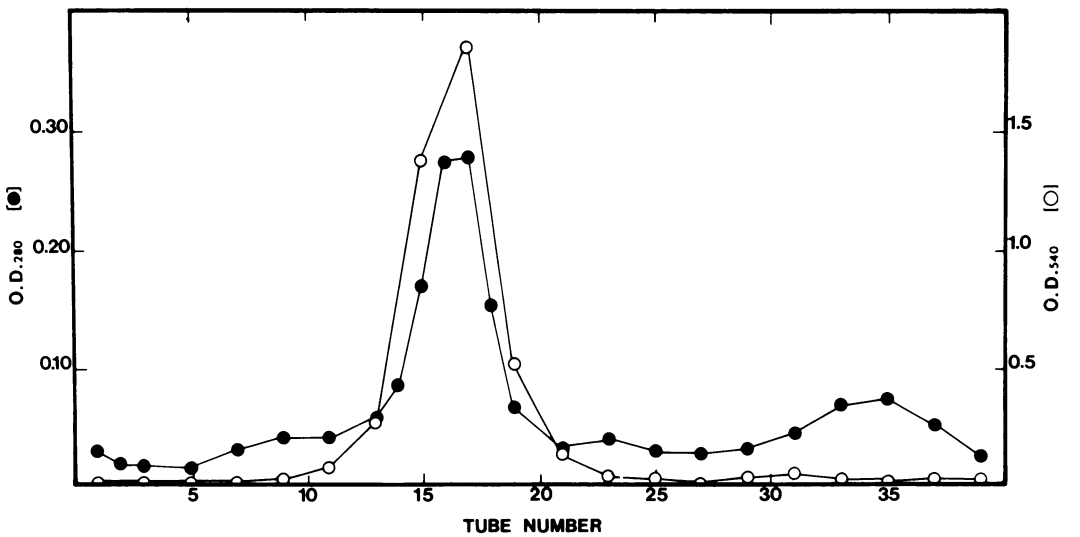

FIG. 3. *Sucrose density gradient fractionation of Anabaena sp. CA glutamine synthetase. The volume of each fraction was 1 ml. Enzymatic activity is expressed in terms of the optical density at 540 nm (OD_{540}) after 20 min in the transferase assay.*

TABLE 1. *Purification of glutamine synthetase from Anabaena sp. CA*

Step	Total protein (mg)	Total units[a]	Transferase sp act (U/mg)	Biosynthetic sp act (U/mg)	Yield[a] (%)
1. Crude extract	984	1,341	1.4		(100)
2. $(NH_4)_2SO_4$ fractionation	110	1,709	15.6	0.3	127
3. Diethylaminoethyl-cellulose	9.3	579	62.5	1.6	43
4. Sucrose gradient	3.0	230	77.8	2.2	17

[a] Based on transferase activity.

tions used. The yield after $(NH_4)_2SO_4$ fractionation was variable in different preparations and therefore could not be taken as evidence for the presence of inhibitors of glutamine synthetase in crude extracts. The ratio of the absorbance at 280 nm to the absorbance at 260 nm for the purified protein was 1.8.

High blank values precluded the use of the biosynthetic activity with crude extracts, due probably to the presence of an active adenosine triphosphatase. The biosynthetic activity of glutamine synthetase is low, as has been previously reported for *A. cylindrica* (2). After sucrose density gradient centrifugation, the homogeneous enzyme had a biosynthetic specific activity of only 2.2 U/mg of protein; however, the ratio of biosynthetic to transferase specific activity remained relatively constant throughout the purification (Table 1).

Molecular weight studies. The molecular weight of the native protein was estimated by chromatography on a Bio-Gel A-1.5m 200- to 400-mesh column having a void volume of 152 ml, as determined by the exclusion of blue dextran 2000. The glutamine synthetase activity peak eluted between thyroglobulin and fructose 6-phosphate kinase, corresponding to an average molecular weight of 592,000. This molecular weight corresponds to that found for the enzyme from other procaryotic sources (1, 7, 31, 33). Electrophoresis of *Anabaena* sp. CA glutamine synthetase in the presence of sodium dodecyl sulfate yielded only one detectable band (Fig. 5), further evidence for the homogeneity of this protein. The subunit molecular weight was determined by comparison to standards of known molecular weight, subjected to simultaneous sodium dodecyl sulfate electrophoresis. An approximate molecular weight of 50,000 for the glutamine synthetase subunit was obtained.

DISCUSSION

Increased interest in the cyanobacteria has focused mainly on their ability to incorporate atmospheric nitrogen. The molecular basis for the control of nitrogen fixation as well as the regulation of subsequent ammonia assimilation has gone largely unexamined in the cyanobacteria. Ammonia assimilation by the action of glutamate dehydrogenase is unlikely since this enzyme has been shown to be absent or present in very low amounts in the cyanobacteria (5, 17). However, glutamine synthetase and glutamate synthase have been shown to be active in the cyanobacteria (3, 10). Evidence that, indeed, these two enzymes function in ammonia assimilation under nitrogen-fixing conditions has been obtained with ^{13}N-labeled nitrogen gas (27, 32). In these studies, glutamine and glutamate were found to be the first labeled organic products formed seconds after cells incorporated atmospheric nitrogen (27, 32). Similar to results of earlier experiments with *A. cylindrica* (2), cell extracts of *Anabaena* sp. CA were found to possess high glutamine synthetase activity when cells were grown under nitrogen-fixing conditions. Glutamine synthetase represents nearly 2% of the total soluble protein in extracts from nitrogen-grown cells. The fact that glutamine synthetase comprises such a large portion of the soluble protein demonstrates its importance to the cell under nitrogen-fixing conditions. Combined nitrogen in the form of NH_4Cl may result in repression of the synthesis of glutamine synthetase, although this has not been rigorously shown as yet. Ammonia repression of glutamine synthetase, however, has been shown in many bacterial systems (1, 6, 7, 18) and perhaps in *A. cylindrica* (2). When nitrate is the nitrogen source, no effect on the levels of glutamate synthetase is seen when compared with those of extracts of cells grown under nitrogen-fixing conditions. Such results are similar to those found with *Pseudomonas aeruginosa* (13), and may indicate that the action of nitrate differs from the effect seen with ammonia once the nitrate enters the cell and is metabolized. Indeed, since the reduction of nitrate is presumably the growth-limiting step, any ammonia produced is probably rapidly assimilated and hence is not available to affect the synthesis of glutamine synthetase.

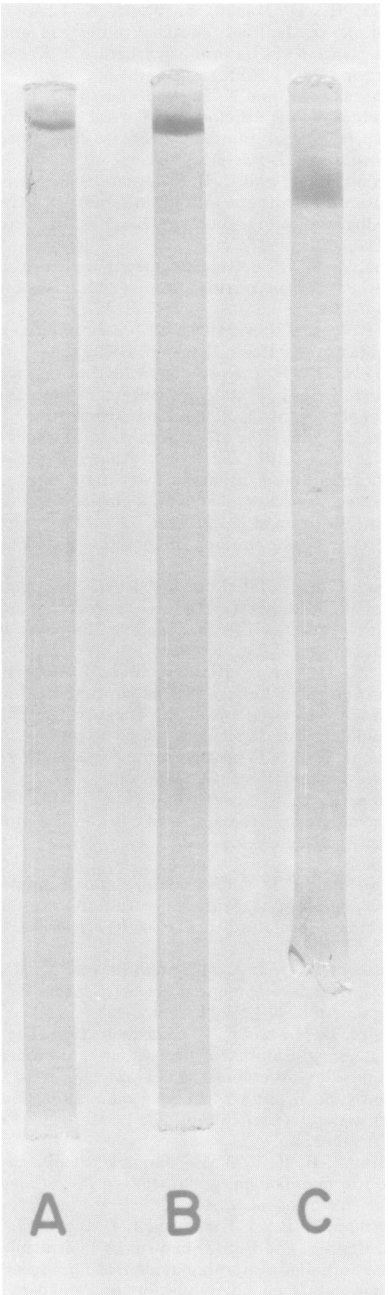

FIG. 4. *Polyacrylamide gel electrophoresis of homogeneous glutamine synthetase: (A) 10 μg of protein on a 7.5% gel; (B) 20 μg of protein on a 7.5% gel; (C) 20 μg of protein on a 6.0% gel.*

Glutamine synthetase from many procaryotic sources has been shown to be covalently modified in cells grown to stationary phase (6, 8, 28). It is for this reason that only late-logarithmic-phase cells were used as a source of the enzyme. However, subsequent experiments designed to show the presence of adenylation in enzyme extracts from ammonia- and light-limited cells have proven negative.

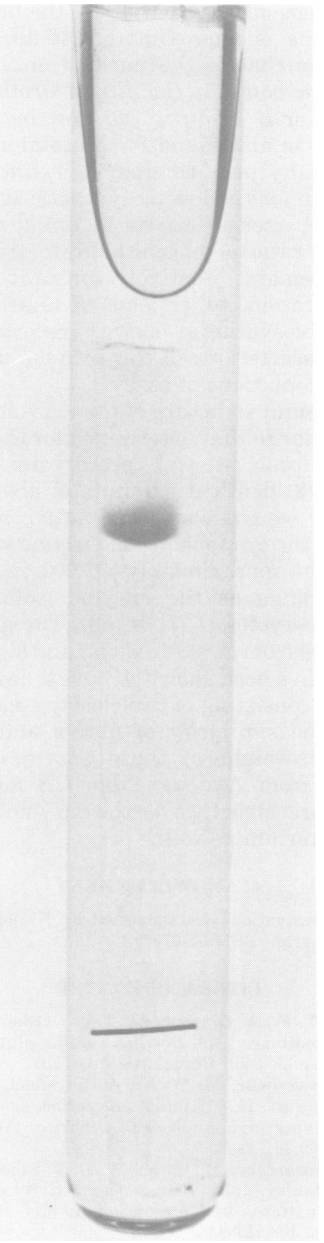

FIG. 5. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Anabaena sp. CA glutamine synthetase. A sample of enzyme containing 20 μg of protein was treated and subjected to electrophoresis in 10% acrylamide as described in the text.*

The purification of glutamine synthetase reported in this investigation was accomplished by a relatively rapid and facile method. The transferase specific activity of the homogeneous enzyme compares favorably with that of the purified enzyme from many sources (12, 14, 16, 33). The biosynthetic activity of the homogeneous enzyme is approximately 10 to 40 times lower than that of the purified enzyme from *Escherichia coli* (33), *Bacillus stearothermophilus* (31), or *B. subtilis* (1). Low biosynthetic activity was also found for glutamine synthetase partially purified from *A. cylindrica* (2). The reason for the low biosynthetic activity for the cyanobacterial enzyme is unknown. However, the ratio of biosynthetic to transferase activity remains relatively constant throughout purification, which leads us to believe that the low biosynthetic activity reported is an actual characteristic of this enzyme under the reported conditions of assay.

The subunit structure of the enzyme appears to be similar to that determined for the enzyme isolated from several procaryotic sources, namely, 12 identical subunits of about 50,000 molecular weight each. The native molecular weight of the cyanobacterial enzyme was determined to be approximately 592,000, in the same general range as the enzyme isolated from other procaryotes (1, 7, 31, 33). The glutamine synthetase from *B. subtilis* (31) and *Escherichia coli* (33) have been shown to have a dodecameric structure consisting of two closely apposed hexagons. The similarity of native and subunit molecular weight of these enzymes to that obtained from *Anabaena* sp. CA suggests a dodecameric structure for the enzyme from this cyanobacterium as well.

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