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THE PURIFICATION OF GLUTAMINE SYNTHETASE FROM *AZOTOBACTER* AND OTHER PROCARYOTES BY BLUE SEPHAROSE CHROMATOGRAPHY

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Summary

We report the facile purification of glutamine synthetase (L-glutamate: ammonia ligase (adenosine 5′-diphosphate-forming), EC 6.3.1.2) in both the adenylylated and unadenylylated form, from *Azotobacter vinelandii* ATCC 12837. A general affinity column, which used as an affinity ligand Reactive blue 2 dye (Cibacron blue) covalently linked to Agarose, was employed as an efficient first step of purification. Further purification to electrophoretic homogeneity employed DEAE-cellulose chromatography and an additional Affigel chromatographic step. The method was used successfully to prepare glutamine synthetase from *Escherichia coli*, *Rhodopseudomonas sphaeroides* and *Anabaena* sp. strain CA.

Introduction

Glutamine synthetase (L-glutamate:ammonia ligase (adenosine 5′-diphosphate-forming), EC 6.3.1.2) is a key regulatory enzyme of nitrogen metabolism in many organisms (for review, see Ref. 1). Shapiro and Stadtman [2] have suggested several physiologically significant mechanisms affecting both synthesis and catalytic activity of the enzyme from *Escherichia coli*. Moreover, the enzyme has been implicated in the control of synthesis of enzymes of nitrogen metabolism [1,3]. It is thus not surprising that glutamine synthetase from a variety of organisms is being investigated in great depth.

Azotobacter vinelandii is an organism that has been well exploited in studies concerned with the regulation of nitrogen fixation. From previous work, an

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obvious locus of control might be glutamine synthetase. However, reliable kinetic information, effector studies, and physicochemical characterization require purified enzyme. The procedure of Woolfolk et al. [4] required extensive modification and gave low yield with the *Azotobacter* enzyme. The zinc precipitation method of Miller et al. [5], when applied to extracts of *A. vinelandii*, performed unreliably in our hands. Blue Sepharose (Affigel Blue), however, was found to bind the enzyme in crude extracts. Glutamine synthetase could then be eluted specifically from the column with ADP; typically 85—95% of the units applied could be recovered, with some fractions over 100-fold purified. This column serves as a first step in a rapid three step procedure which results in relatively high yields of enzyme from a variety of organisms, without resorting to potentially damaging protein precipitation techniques.

Materials and Methods

Affigel Blue (100—200 mesh Blue Sepharose) was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.); imidazole, L-glutamine, and the sodium salts of L-glutamic acid, ADP, ADP, ATP, NADPH, and other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); DEAE-cellulose (DE-52) was purchased from Whatman (Clifton, NJ, U.S.A.); acrylamide and *N,N'*-methylene-bisacrylamide were obtained from Eastman Kodak Co. (Rochester, NY, U.S.A.); all other compounds were reagent grade.

Organisms and growth conditions. Burk's nitrogen-free salts [6] served as a base for *Azotobacter* media. Cells of *A. vinelandii* ATCC 12837 were grown at 33°C as described [7] and harvested at stationary phase of growth. If adenylylated enzyme was desired, 10 mM ammonium acetate was added to the culture 10 min prior to harvesting. *E. coli* K 12 (strain 2eo1c) was grown to stationary phase at 37°C in the minimal medium of Howard-Flanders et al. [8] (supplemented with 0.1% each of glutamate, threonine and leucine, for which our strain was auxotrophic). *Rhodopseudomonas sphaeroides* 2.4.1 Ga was grown photoheterotrophically with 0.6% malate and 0.2% glutamate in the medium of Ormerod et al. [9] as previously described [10]. *Anabaena* sp. CA was grown as described by Stacey et al. [11]. *Saccharomyces cereviseae* was grown in Burk's salts with 0.5% glucose, 0.2% glutamate and 0.5% yeast extract. The pH of the medium was adjusted to 6.0 and the cultures were aerated at 33°C until stationary phase growth was reached.

Preparation of cell-free extracts. All organisms (except *Anabaena* CA) used in this study were subjected to the same harvesting and treatment protocol. Cells were washed in buffer A and extracts prepared by passing suspensions twice through a French pressure cell at 20 000 lb/inch^2 in the presence of DNAase. The extracts were centrifuged at 100 000 $\times g$ for 1 h and the supernatant fluid dialyzed against 200 vols. buffer A, with 3—4 changes of buffer, for 24 h at 5°C.

Protein determinations. Protein was estimated routinely by the method of Lowry et al. [12] (Fraction V of bovine serum albumin was used as a standard) and occasionally by the method of Layne [13].

Enzyme assays. Glutamine synthetase was assayed during purification by the γ-glutamyltransferase reaction using the procedure of Shapiro and Stadtman

[14] with the following modifications: the pH of the double-strength reaction mixture was adjusted to 7.55 with 2 M KOH instead of NaOH. The reaction was allowed to proceed for 10 min at 33°C; linear production of γ-glutamyl hydroxamate was observed for up to 30 min under our standard assay conditions. Corrections for glutaminase activity in crude extracts were accounted for by preparing blanks minus ADP and potassium arsenate [15]. In all cases this activity was shown to be negligible. A unit is defined as the amount of enzyme required to produce 1 μmol γ-glutamyl hydroxamate/min. Specific activity is expressed as units/mg protein. Biosynthetic activity was determined for purified and partially purified preparations of the enzyme according to the method of Shapiro and Stadtman [14]. Linearity with time was demonstrated over a range of 0—0.25 μmol inorganic phosphate produced.

The adenylylation state of the *Azotobacter* enzyme was determined by comparison of transferase activity in the presence and absence of 60 mM $MgCl_2 \cdot 6\ H_2O$ after the method of Stadtman et al. [16].

Blue Sepharose chromatography. Two columns of Affigel Blue were used. For preparation of large quantities of *A. vinelandii* enzyme, the dialyzed extracts were applied to a 65 ml (2.7 × 12 cm) column with up to 3 g total protein without overloading the column. A 5 ml column was used for the first step in purification from extracts of 1 or 2 l cultures. It also served as a final step to remove remaining contaminating proteins from the pooled DEAE-cellulose fractions. The crude extract was applied to the column and the column washed with buffer A until the absorbance at 280 nm of the eluate was 0.01 or less. Two column volumes of 2 mM ADP in buffer A were used to elute glutamine synthetase from the column.

DEAE-cellulose chromatography. Whatman DE-52 DEAE-cellulose was equilibrated with buffer A and a 20 ml column was poured. Pooled, high specific-activity fractions from the Affigel column were applied to this column. At least 500 ml of buffer A were used to wash the enzyme bound to the cellulose. A 200 ml 0.1 M KCl wash removed some contaminating protein; a 400 ml 0.1—0.5 M linear KCl gradient was then used to elute glutamine synthetase as well as to separate other contaminants from the enzyme.

Diaflo ultrafiltration. Fractions with high transferase activity from the DEAE-cellulose column were pooled and concentrated by pressure filtration (nitrogen) using an Amicon ultrafiltration apparatus with a Diaflo XM-100 membrane.

Polyacrylamide gel electrophoresis. Standard, cylindrical 7% gels were prepared by the method of Davis [17] and stained with 0.04% Coomassie brilliant blue G-250 in 3% $HClO_4$; destaining was done with 10% acetic acid. SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli [18]. Gels were stained with 0.04% Coomassie brilliant blue R-250 in 10% acetic acid/25% isopropanol and destained with acetic acid/10% isopropanol.

Results

Since the purification process involved the use of a generalized affinity gel, we investigated the specificity of elution from the column. *Azotobacter* extract

was applied to the 5 ml column and elution attempted with two-column volumes of 5 mM concentrations of each of the following: KCl, glutamate, glutamine NADPH, AMP, and ATP. 40—50 ml buffer were used to wash the column following each elution treatment. Fig. 1 shows that only ATP was effective in eluting active glutamine synthetase. Increases in absorbance at 280 nm, of course, are expected when NADPH, AMP, and ATP are employed. In a separate experiment a 200 ml linear KCl gradient (0—1.0 M) was used to attempt elution of glutamine synthetase from the Affigel column. Although some protein was removed from the column by this salt gradient, no glutamine synthetase activity was detected in the eluate. The application of 2 mM ADP eluted 90% of the units applied.

The Affigel chromatographic elution profiles of glutamine synthetase from a variety of organisms are presented in Fig. 2. The profiles are similar to each other with the activity peak being manifest early in the rise in A_{280} and trailing into fractions collected after all of the ADP had been applied to the column. Extracts of *Anabaena* sp. CA were prepared by the method of Stacey et al. [11] and the elution profile shown is that for partially purified enzyme.

An extract of *S. cereviseae*, with a transferase-specific activity of 0.2 unit/mg of protein, was also applied to the small Affigel column. It passed through the column without binding under conditions identical to those which permitted the binding of the other enzymes. The glutamine synthetase of *S. cereviseae* thus differs from the procaryotic enzymes we've used in that no binding occurs under our standard conditions.

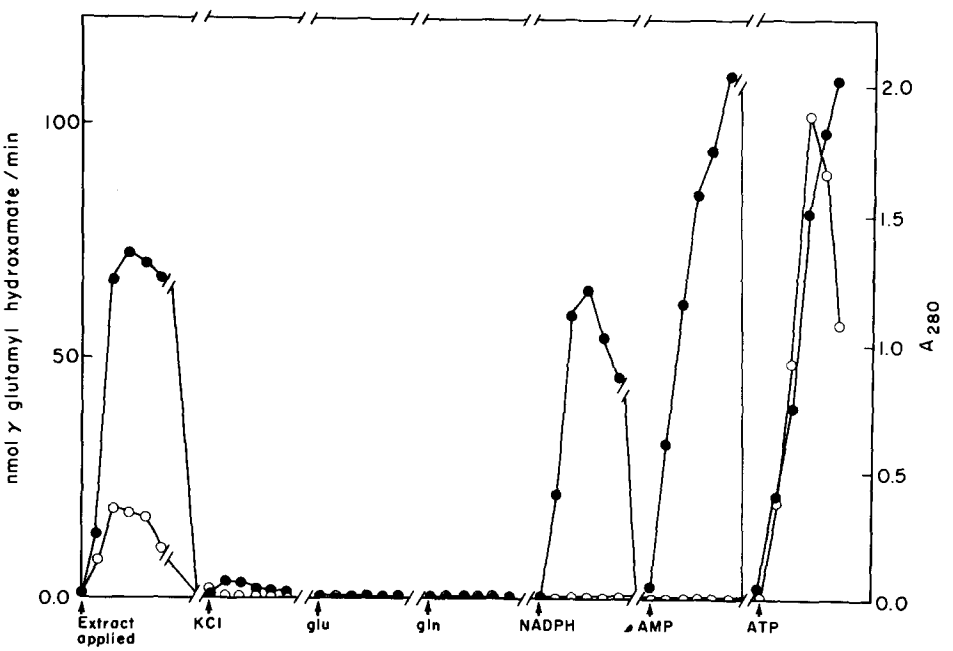

Fig. 1. Elution of *Azotobacter* glutamine synthetase from an Affigel (1.0 X 6.0 cm) column. Each of the compounds used was applied at a concentration of 5 mM in two column volumes of buffer A. Glutamine synthetase is expressed in terms of transferase activity, nmol γ-glutamyl hydroxamate produced after a 10 min assay of 10-μl samples (○———○); (●———●), A_{280}. The column was washed with buffer A between applications of each eluent.

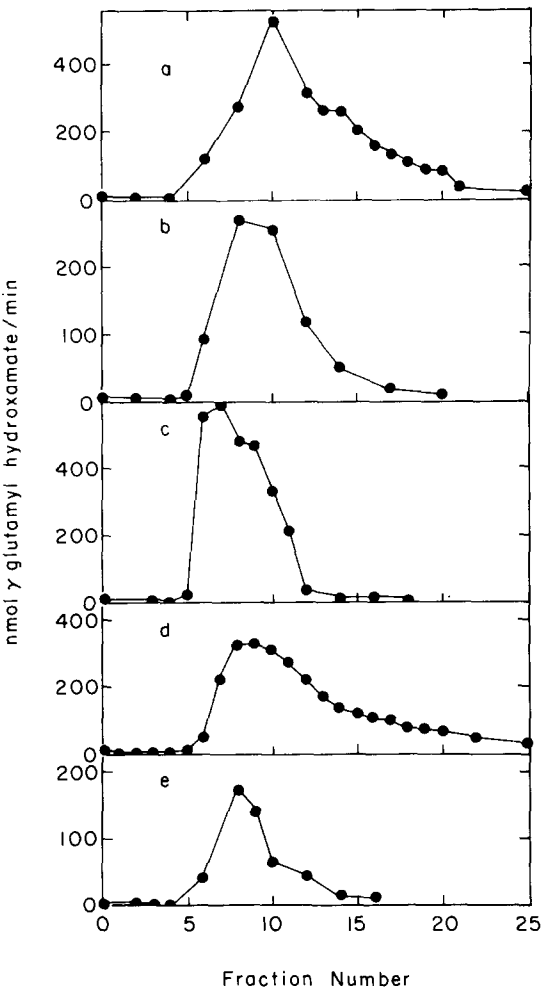

Fig. 2. Affigel chromatographic elution profiles of glutamine syhthetase from (a) *A. vinelandii*, $\bar{n} = 0$; (b) *A. vinelandii*, $\bar{n} = 10.6$; (c) *E. coli*; (d) *Anabaena* sp. CA; (e) *R. sphaeroides*. Crude extracts were dialyzed in buffer A, applied to the column and washed with buffer A until the eluate A_{280} was 0.01. Elution was effected with 2 mM ADP.

DEAE-cellulose anion-exchange chromatography was performed on the pooled peak fractions from the first large Affigel column for further purification of the *Azotobacter* enzyme. A broad second A_{280} peak, eluting in the range of 0.35—0.40 M KCl, showed high transferase activity. These fractions were pooled and subjected to ultrafiltration; this provided a rapid means of removing the KCl and placing the enzyme back into the standard buffer A environment, permitting comparable assays and protein determinations. At this point in the purification scheme, polyacrylamide gel electrophoresis revealed trace contamination by more-rapidly migrating proteins (Fig. 3). The protein could be rendered electrophoretically homogeneous by an additional Affigel step using the 5 ml column. Elution characteristics of this partially purified

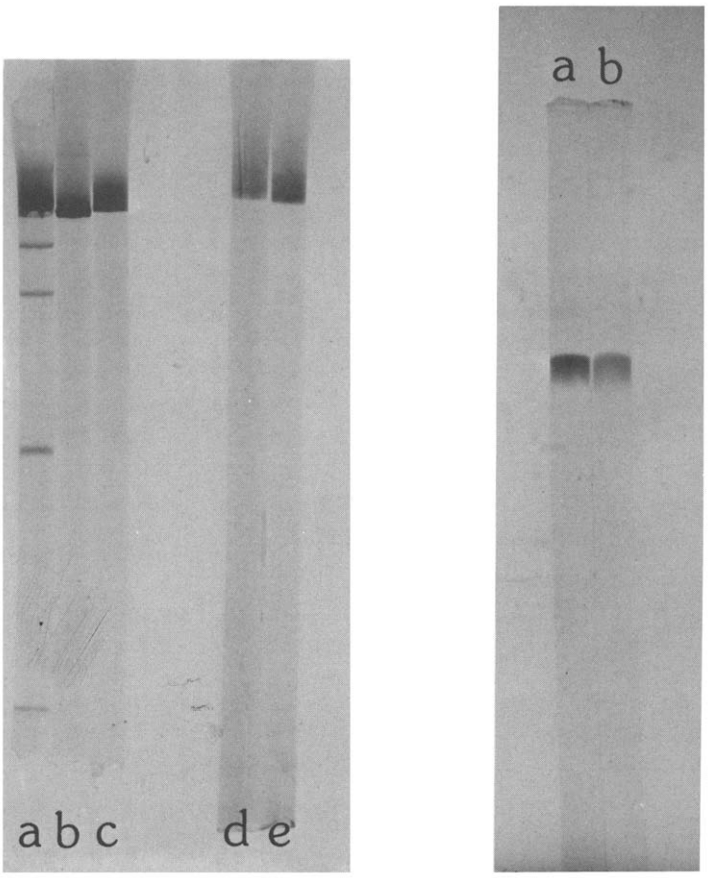

Fig. 3. Polyacrylamide gel electrophoretograms of the *Azotobacter* enzyme at various stages of purity: (a) after first Affigel column; (b) after DEAE-cellulose chromatography and Diaflo concentration, and (c) after the second Affigel column. 50 μg protein were applied to each gel; after electrophoresis, gels were stained with Coomassie blue and destained. Gels (d) and (e) are activity stains of the same stage of purity as in gels (b) and (c), respectively. Activity stains were performed by incubating the gels in the transferase reaction mixture for 30 min at 33°C and then treating them with $FeCl_3$/trichloroacetic acid stop mixture to detect the γ-glutamyl hydroxamate produced.

Fig. 4. SDS-polyacrylamide gel electrophoretograms of the adenylylated (a) and the unadenylylated (b) glutamine synthetase from *A. vinelandii*.

enzyme were the same as those observed for the crude extracts.

Polyacrylamide electrophoretograms of *Azotobacter* enzyme fractions from each of these purification steps are presented in Fig. 3. Also shown are activity stains of parallel gels.

SDS-polyacrylamide gel electrophoretograms of the adenylylated and unadenylylated *A. vinelandii* enzyme show one band which migrates at a distance consistent with a subunit size of about 55 000 daltons (Fig. 4).

During the purification of adehylylated glutamine synthetase of *A. vinelandii*, the average adenylylation state ($\bar{n}$), as determined by the magnesium sensitivity of transferase activity, did not change significantly. An $\bar{n}$ of 10.9 was determined for the crude extract preparation and this value did not fall below

TABLE I

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56 g cells were used. Specific activity is of the γ-glytamyl transferase activity. Average adenylylation state was determined by comparison of the transferase activity in the presence and absence of 60 mM $MgCl_2$ after the method of Stadtman et al. [16]. The values represent the average number of the 12 subunits which are adenylylated. Fractions from the first Affigel column varied in n over a range of 10.6—11.4; the values shown above are those of pooled fractions.

Fractions	Volume (ml)	Total units	Total protein (mg)	Specific activity	Yield (%)	Adenylylation ($\bar{n}$)
Dialyzed crude extract	48	1021.7	1277.2	0.8	100	10.8
First Affigel fraction	65	900.2	28.6	31.5	88	11.4
DEAE-cellulose (Diaflo-concentrated pooled fractions)	30	346.7	3.6	96.3	34	10.4
Second Affigel fraction	15	306.5	3.2	95.8	30	10.6

10.4 nor above 11.3 throughout the purification; it is likely that these fluctuations are within the limits of experimental error.

The biosynthetic activity of the best preparations of unadenylylated glutamine synthetase from *A. vinelandii* was about 30 μmol of phosphate produced/min per mg protein, but typical values were 22—25 μmol/min per mg protein. A summary of the purification and recovery of adenylylated glutamine synthetase from Azotobacter is shown in Table I.

The general utility of the method is also exemplified by the fact that the glutamine synthetase of *E. coli* was purified about 90-fold using the first Affigel column and the DEAE-cellulose column. A crude extract transferase activity of 0.82 unit/mg protein was increased to 73.8 units/mg protein. After passage through the first Affigel column and the DEAE-cellulose column, the glutamine synthetase from *R. sphaeroides* showed a transferase activity of 102.8 which represents a 66-fold purification of the activity over that of the high-speed supernatant fluid or about 100-fold over that of the low-speed supernatant fluid. Recoveries were 44% for the enzyme from *E. coli*, and 45% for the *R. sphaeroides* enzyme.

Discussion

Affigel Blue is a general affinity gel which is synthesized by coupling Cibacron blue F3GA, a reactive blue dye, to cross-linked agarose beads. The gel is specific for nucleotide-requiring enzymes, perhaps due to the fact that the dye binds to the dinucleotide fold common to these proteins [19,20]. We have shown that the Affigel column is a highly efficient first step for the purification of glutamine synthetase from diverse procaryotic sources. The column operates in a highly specific manner with regard to the binding of glutamine synthetase and it does not function as an ion-exchange column since up to 1 M KCl failed to elute the *Azotobacter* enzyme from the gel. However, 88—97% of the units applied to the column was eluted by either 2 mM ADP or ATP. Certainly since ADP serves as an activator of the transferase activity [2] and ATP serves as a substrate for the biosynthetic reaction [2] and has been shown to bind to the

enzyme [21,22], it is not surprising that these nucleotides elute the bound glutamine synthetase. AMP failed to elute the enzyme in spite of the fact that it has been demonstrated to be a negative effector for the enzymes of *E. coli* [23], *Anabaena* (Ref. 24, Stacey, G. and Tabita, F.R., unpublished data), and *A. vinelandii* (Lepo, J.E., unpublished data).

We have found that up to 3.5 g crude extract protein could be loaded onto the 65 ml column with no detectable activity in the first 100 ml buffer wash. Furthermore, the pooled fractions of the glutamine synthetase peak comprise as much as 95% of the total units applied to the column. ATPase activity of crude extracts make accurate biosynthetic assay impractical, however peak fractions of the first Affigel column could be exhaustively dialyzed (to remove ADP) and rendered suitable for biosynthetic assay.

Anabaena sp. strain CA extracts were not applied directly to the Affigel column. The elution profile shown is that of enzyme that had been partially purified. High levels of ribulose 1,5-bisphosphate carboxylase are present in the organism under the conditions used for growth. Ribulose 1,5-bisphosphate carboxylase will bind to the column and overload it such that much of the glutamine synthetase will not bind and will be lost in the preliminary wash. In *R. sphaeroides*, the problem was averted by growing the organism photoheterotrophically on a malate/glutamate medium. This results in repression of the carboxylase [10] and also results in the induction of significant levels of glutamine synthetase (Ref. 25, and Lepo, J.E., unpublished results). There seemed to be no impairment of the function of the Affigel column when used with extracts of this organism. About 50% of the glutamine synthetase of *R. sphaeroides* was in the pellet following the 100 000 × *g* centrifugation, but since the supernatant fluid showed 50% higher specific activity than the resuspended pellet, it was used in the further purification of the *R. sphaeroides* enzyme. It should be noted that, Johansson and Gest [25] have used sedimentation at 140 000 × *g* for 2 h as a step in the partial purification of the glutamine synthetase from the related organism, *Rhodopseudomonas capsulata.*

The Affigel Blue column seems to present a powerful tool for the purification of glutamine synthetase from a variety of organisms when it is used in conjunction with other protein purification techniques. It offers the advantages of being rapid, gentle, and highly efficient, giving a high yield of the desired protein.

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