Regulation of Glutaminase B in *Escherichia coli*

I. PURIFICATION, PROPERTIES, AND COLD LABILITY

(Received for publication, January 27, 1975)

STANLEY PRUSINER,* JOE N. DAVIS, AND E. R. STADTMAN

From the National Institutes of Health, National Heart and Lung Institute, Laboratory of Biochemistry, Bethesda, Maryland 20014

*Escherichia coli* contains two glutaminases, A and B, with pH optima below pH 5 and above pH 7, respectively. Neither glutaminase A nor B is released from *E. coli* by osmotic shock. Glutaminase B has been purified 6,000-fold and the purified preparation is estimated to contain about 40% glutaminase B. The enzyme has a molecular weight of 90,000 and an isoelectric point of 5.4. Glutaminase B exhibits a broad pH optimum between 7.1 and 9.0. Only L-glutamine is deamidated by glutaminase B, L-asparagine and D-glutamine are not deamidated. The substrate saturation curve for glutaminase B shows an intermediary plateau region.

Like many regulatory enzymes, glutaminase B is cold-labile. The enzyme is inactivated by cooling and activated by warming; both processes are first order with respect to time. The activation energy for activation by warming was calculated to be 5900 cal/mol. Activation by warming increased the $V_{max}$ and decreased the $K_m$ for L-glutamine, but did not alter the molecular weight of the catalytically active enzyme. Borate and glutamate protected glutaminase B from inactivation by cold.

Glutamine has a central role in cellular nitrogen metabolism (1). The intracellular levels of glutamine are determined by the rates of enzymatic synthesis and degradation. Glutamine synthetase catalyzes the synthesis while glutaminases catalyze the hydrolytic degradation of glutamine, as shown in Reactions 1 and 2, respectively.

$$\text{L-Glutamate} + \text{NH}_3 + \text{ATP} \rightarrow \text{L-glutamine} + \text{ADP} + \text{P}_i \quad (1)$$

$$\text{L-Glutamine} + \text{H}_2\text{O} \rightarrow \text{L-glutamate} + \text{NH}_3 \quad (2)$$

Two other classes of enzymatic reactions also participate in the degradation of glutamine to glutamate, as shown in Reactions 3 and 4.

Biiosynthetic precursor + L-glutamine $\rightarrow$

biosynthetic intermediate or product + L-glutamate

$$\text{a-Ketoglutarate} + \text{L-glutamine} + \text{NADPH} \rightarrow$$

$$2\text{L-glutamate} + \text{NADP} \quad (4)$$

A variety of enzymes catalyze the incorporation of the amide nitrogen of glutamine into biosynthetic end products or intermediates; in some cases these biosynthetic reactions require ATP. The last enzymatic Reaction 4 is unique to prokaryotes and involves the donation of the amide nitrogen of glutamine to a-ketoglutarate with the concomitant oxidation of NADPH (2).

Reactions 3 and 4 depend on the availability of a second and sometimes third substrate, in contrast to Reaction 2, which depends only on L-glutamine, since H$_2$O is probably in abundant supply intracellularly. In *Escherichia coli*, Reaction 2 is catalyzed by glutaminases A and B (3, 4).

Glutaminase A, which is active only at acid pH, has been previously characterized (3, 5). The regulation of glutaminase B is of considerable interest because its action directly opposes that of glutamine synthetase. Since both of these enzymes are active at neutral pH, their coupling would result in a “futile cycle” of amide synthesis and degradation (4, 6).

In this communication we report on the purification and some of the properties of glutaminase B from *E. coli*. Subsequent papers give detailed information on the regulation of glutaminase B by cellular metabolites.

METHODS

Materials—$L-\left[U-^{14}C\right]$Glutamine was obtained from New England Nuclear and purified by chromatography over Dowex 1-C1. Agarose was purchased from Bio-Rad, and acrylamide and bisacrylamide obtained from Canalco. The bis(2-hydroxyethyl)-imino-tris(hydroxymethyl)methane was obtained from General Biochemicals while the N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid was purchased from Eastman. Sodium cacodylate was purchased from Sigma and the acid obtained after passage over a column of Dowex 50 (H$^+$).

Bacterial Growth—*Escherichia coli* B (wild type Oregon) was a generous gift from Dr. Martin Gellert. The bacteria were grown at 37° with vigorous aeration over a minimal medium limited in glucose containing 1.7 mm MgSO$_4$, 43 mm NH$_4$Cl, and 44 mm K$_2$SO$_4$, 100 mm potassium phosphate, pH 7.4, 100 mm NH$_4$Cl, and 44 mm glucose. The cells were harvested in early stationary phase and frozen in liquid nitrogen. Fermentations of 350 and 1000 liters gave approximately 2 and 8 kg, respectively, of frozen cell paste. The pH was maintained at

* Present address, Departments of Neurology and Biochemistry and Biophysics, University of California, School of Medicine, San Francisco, Ca. 94143.
Assay Procedures—Glutaminase activity was determined by measuring the formation of labeled glutamate from L-[U-14C]glutamine at 37°C for 5 min (7). Glutaminase A assay solution (0.1 ml) contained 50 mM Tris and 0.1% sodium acetate (pH 5.5); glutaminase B assay solution (0.1 ml) contained 30 mM L-glutamate and 100 mM potassium phosphate (pH 7.1). Blanks contained no enzyme. All assays using highly purified preparations of glutaminase B also contained bovine serum albumin (0.5 mg/ml). The L-glutamate was isolated by chromatography on Dowex 1-C1 and the radioactivity measured using Bray's solution with a Beckman LS 250 liquid scintillation counter. Identical results were obtained when glutaminases A and B were assayed measuring the NH₃ produced (8) under the above assay conditions.

A coupled spectrophotometric assay measuring the rate of L-glutamate formation from L-glutamate at neutral pH was also used. The reduction of AcPyAD at 37°C was measured at 383 nm (9). The assay mixture (1.0 ml) contained 20 mM L-glutamate, 100 mM potassium phosphate (pH 7.0), 500 ug of bovine glutamate dehydrogenase in 50% glycerol, and 0.15 mM AcPyAD. The assay was very convenient but was linear with enzyme concentration in a relatively narrow range in contrast to the radioisotopic assay which was linear with time and enzyme concentration over a broad range.

In addition, glutaminase activity was measured by the formation of γ-glutamyl hydroxamate from L-glutamate and NH₃OH. The assay solution (0.5 ml) contained 100 mM imidazole-HCl, pH 8.3, 30 mM L-glutamate, and 500 mM NH₃OH (10). The reaction was stopped and the color developed by the addition of 1.5 ml of 3.5% FeCl₃, 2% trichloroacetic acid, and 0.25 N HCl. The absorbance was measured at 540 nm in a Gifford 240 spectrophotometer.

Alkaline phosphatase activity was determined by measuring the release of p-nitrophenol from p-nitrophenyl phosphate (11). Protein was measured by the biuret method using crystalline bovine serum albumin as standard unless otherwise noted (12).

Osmotic Shock—Osmotic shock of E. coli was performed on logarithmically growing cells (13). The cells were washed by centrifugation (3000 x g) three times in 10 mM Tris-Cl, and 30 mM NaCl, pH 8.0 at 4°C. The cells were then resuspended in 33 mM Tris-Cl, pH 8.0, and 40% sucrose. EDTA (0.4 mM) was added and the cells incubated at 23°C for 3 min. The cells were then centrifuged and the supernatant concentrated by ultrafiltration using an Amicon UM-10 membrane.

Preparative Electrophoresis—A Camaco preparative electrophoresis column, 2.6 cm in diameter, was used with a discontinuous polyacrylamide gel system. The electrophoresis was performed at pH 7.8 using the system of Rodbard and Chrambach designated "D" (14). The 10% separating gel contained a 30:1 ratio of acrylamide to bisacrylamide and 0.107 M bis-Tris. Potassium persulfate (0.055 mM) and Dimethyl (6.54 M) catalyzed polymerization of the gel. The 3.5% upper gel contained 20% acrylamide to bisacrylamide, and 0.002 M bis-Tris adjusted to pH 6.28 with cacodylic acid. Photoactivated riboflavin (0.013 mM) and Temed (3.25 M) catalyzed polymerization of the gel. The 3.5% upper gel contained 20% acrylamide to bisacrylamide, and 0.002 M bis-Tris adjusted to pH 6.28 with HCl.

Analytical Electrophoresis—Analytical polyacrylamide electrophoresis was carried out in the bis-Tris, pH 7.8, system designed "D" of Rodbard and Chrambach (14) and in the Tris glycine, pH 9.6, system of Ornstein and Davis (15). In both systems the gels contained a 30:1 ratio of acrylamide to bisacrylamide and only the separating gel was used. To prevent persulfate oxidation of the sample, a current of 0.5 mA/tube was applied to the gels for 20 min before sample application and 0.24 mM thyroglobulin was added to the upper buffer. The samples containing 100 to 200 ng of protein in 20% sucrose which contained the tracking dye bromphenol blue. Electrophoresis was conducted for 1 to 2 hours with a constant current of 2 to 4 mA/tube at room temperature or 4°C. Upon completion of electrophoresis, gels were stained for protein with Amido Schwarz or Coomassie blue dye and for glutaminase with nitroblue tetrazolium dye. Staining for glutaminase was accomplished by coupling the formation of glutamate with the reduction of the tetrazolium dye using glutamic dehydrogenase (16).

The abbreviations used are: bis-Tris, bis(2-hydroxymethyl)-iminotris(hydroxymethyl) methane; Temed, N,N,N',N'-tetramethylethylenediamine; Tes, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; cAMP, cyclic AMP, adenosine 3'-5'-monophosphate.

Electrophoresis on cellulose acetate strips was performed as described by Kohn (17).

RESULTS

Cellular Localization of Glutaminases

Distribution of glutaminases A and B in the E. coli cell was determined using the "EDTA osmotic shock" procedure of Weiner and Heppel (20). Cells were grown on the glucose-limited medium described above and on a glycerol low chloride medium used in the isolation of glutamine-binding protein (20). Less than 5% of the total glutaminase A and B activity was released from logarithmically growing cells by EDTA osmotic shock (Table I). The remainder of the activity was found in the soluble fraction of shocked cells after exposure to sonic oscillation. Alkaline phosphatase was used as a marker for periplasmic enzymes and more than 90% of the enzyme was found in the shock fluid. Thus, glutaminases A and B, like glutamine synthetase (21), are not released by osmotic shock. In contrast, glutamine-binding protein is found in the periplasmic space (20).

Growth Conditions

Studies on the regulation of the cellular levels of glutaminases A and B have revealed that glutaminase A levels are controlled by nitrogenous metabolites and cAMP (22) while glutaminase B levels are independent of the nutritional conditions. Details of studies demonstrating the constitutive control of glutaminase B are given in another communication. To date all attempts to influence the level of glutaminase B either by altering the composition of the culture media or by mutagenesis have been unsuccessful.

Purification

Since glutaminase B is a constitutive enzyme and, as shown in C. Caban, unpublished data.

### Table I

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Per cent total enzyme activity measured by osmotic shock</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>Minimal medium</td>
<td>100</td>
</tr>
<tr>
<td>Minimal medium (low chloride)</td>
<td>91</td>
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</tbody>
</table>

Quantitative estimates of protein bands were made by scanning the gels using a modified Zeiss spectrophotometer. Electrophoresis on cellulose acetate strips was performed as described by Kohn (17).

### Molecular Weight Determinations

Molecular weight was estimated by gel filtration using Sephadex G-200 in a column (1.8 x 100 cm) using upward flow at a rate of 7 ml/cm/hour at 4°C. The column was calibrated with known protein standards. Molecular weights were also determined using polyacrylamide gel electrophoresis. The glutaminase B activity was located using the specific enzymic stain described above. Electrophoresis of appropriate standards on separate gels was conducted simultaneously. The molecular weight of glutaminase B was estimated according to the method of Hedrick and Smith (18).

Isoelectric Focusing—Isoelectric focusing was performed in thin polyacrylamide gel slabs essentially as described by Vesterberg (19) but modified by Caban for micromass analysis.\(^1\) This system consisted of polyacrylamide gel layered on cellulose acetate strips which were used for support. pH 3 to 10 ampholyte carrier (LKB Instruments) was used and focusing required approximately 6 hours. The gel was then sliced into sections 1 mm wide, and the pH and enzyme activity determined at each section after addition of 0.2 ml of water.

1\(^{1}\) The abbreviations used are: bis-Tris, bis(2-hydroxymethyl)-iminotris(hydroxymethyl)methane; Temed, N,N,N',N'-tetramethylethylenediamine; Tes, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; cAMP, cyclic AMP, adenosine 3'-5'-monophosphate.
in Table II, comprises a minute fraction of total cellular protein, large scale purification was necessary. The procedure described below is for 30 kg of E. coli cell paste and is essentially the same for preparations starting with 5 kg of cell paste. We are grateful to the New England Enzyme Center for their cooperation and the use of their facilities in the large preparation of the enzyme in Steps I to IV. Except as noted, all operations were carried at 2-4°C.

**Step I: Cell Extracts**—Thirty kilograms of frozen E. coli were blended with 60 liters of 10 mM imidazole-HCl, pH 7.0, in a Gilford-Wood mixing apparatus. For smaller preparations a Waring Blender was used. The suspended cells were stained through cheesecloth and disrupted in a Manton-Gaulin laboratory homogenizer at 8,000 p.s.i. Two passes through the pressure cell of the homogenizer were required. Cellular debris were then removed by two passes through a DeLaval continuous flow centrifuge at 8,000 × g. The pH was adjusted to 7.0 with 2 N NH₄OH. Alternate procedures for the extraction of glutaminase B from _E. coli_ were examined on a small scale. Sonication, grinding with alumina, homogenization with glass beads, alternate freezing and thawing, grinding with dry ice, and lysis with Brij detergent all gave similar results to those obtained with a French pressure cell or Manton-Gaulin homogenizer.

**Step II: Streptomycin Precipitations**—Streptomycin sulfate solution (10%, w/v, pH 7.0) was added slowly to the supernatant of the crude extract which contained 20 to 30 mg/ml of protein. The final concentration of streptomycin was 1%. The mixture was stirred for 30 min and passed through a Sharples type 16 continuous flow centrifuge at 13,000 × g.

**Step III: Ammonium Sulfate Fractionation**—To the supernatant solution from Step II was added streptomycin sulfate to give 28% saturation. The suspension was stirred slowly for 30 min and clarified in an Electro Nuclecels RK continuous flow ultracentrifuge at 90,000 × g. The supernatant was recovered and the pH adjusted to 7.0 with 2 N NH₄OH.

Solid ammonium sulfate sufficient to give 49% saturation was then added with stirring. The suspension was allowed to stand for 1 hour and was then clarified by ultracentrifugation. The pellet was recovered and resuspended in buffer containing 20 mM potassium aspartate, 20 mM Na₂B₄O₇, and 10 mM imidazole-HCl at pH 7.1 to give a final protein concentration of 30 mg/ml.

To this solution, solid ammonium sulfate sufficient to give 36% saturation was added. No correction was made for the residual ammonium sulfate which was negligible. After standing for 1 hour, the suspension was clarified by ultracentrifugation as described above and the pellet resuspended in buffer containing 20 mM potassium aspartate, 20 mM Na₂B₄O₇, 10 mM imidazole-HCl, and 30 mM potassium phosphate, pH 7.1, to give a final protein concentration of 50 mg/ml. In this buffer the enzyme was relatively stable and it was used throughout the remaining steps of purification. The enzyme solution was dialyzed against 20 volumes of the above buffer with two changes of buffer. Total dialysis time was 8 to 12 hours.

**Step IV: DEAE-fibrous Cellulose Chromatography**—With the sample conductivity less than 5.0 mmho, one-half of the sample was absorbed at room temperature to the top of a DEAE-cellulose column (DE23) equilibrated with buffer solution with a conductivity of 5.0 mmho which contained 20 mM potassium aspartate, 20 mM sodium borate, 10 mM imidazole-HCl, and 50 mM potassium phosphate. The sample was either applied directly to the top of the column (13.5 x 50 cm) or adsorbed batchwise to the top 30 cm of the column. Approximately 1 liter of column volume was required for each 20 g of protein to be absorbed. The column was washed with 2 column volumes of the 5.0-mmho buffer. A linear gradient was then developed with the mixing chamber containing the 5.0-mmho buffer and the reservoir chamber containing the same buffer except the conductivity was increased to 30 mmho using 300 mM potassium phosphate. The column had a flow rate of 35 ml/cm²/hour and required approximately 16 hours for completion. Chromatography was at room temperature. Higher recoveries of enzyme activity were obtained on a smaller scale when a column (10 x 20 cm) was pumped at 100 ml/cm²/hour and required only 4 hours for completion. Fractions with greater than 10% of the peak activity were pooled (Fig. 1A). Solid ammonium sulfate was added to give 50% saturation at 4°C and the suspension centrifuged at 10,000 × g. The pellet was resuspended in the 5.0-mmho buffer to give a final protein concentration of 50 mg/ml.

**Step V: Agarose 1.5m Gel Filtration**—Two hundred milliliters of protein solution were then applied to an Agarose 1.5m (100 to 200 mesh) column (5 x 120 cm) at 4°C. The sample was followed by an equal volume of the 5.0-mmho buffer containing 10% sucrose. The column was developed upward and in tandem with another column of identical composition and size. Elution was at a flow rate of 30 ml/cm²/hour with a pressure of 100 cm of H₂O. Those fractions containing greater than 10% of the peak activity were pooled (Fig. 1B).

**Step VI: DEAE-microgranular Cellulose Chromatography**—The pooled sample was then absorbed to the top of a DEAE-cellulose DE32 column (10 x 30 cm) at room temperature which had been equilibrated with the 5.0-mmho buffer described above. One liter of column volume was required for each 2 g of protein to be absorbed. After absorption of the sample, the column was washed with 2 volumes of buffer and the glutaminase B eluted stepwise by elevating the potassium phosphate concentration to 75 mM to give a final conductivity of 7.5 mmho (Fig. 1C). A peristaltic pump was used to achieve a flow rate of 80 ml/cm²/hour. The fractions with greater than 10% of the peak activity were pooled and solid ammonium sulfate was added to give 50% saturation at 4°C. The suspension was centrifuged at 30,000 × g and the pellet was resuspended in the 5.0-mmho buffer to give a final protein concentration of 20 mg/ml.

**Step VII: Preparative Electrophoresis**—Five milliliters of the above protein solution (100 mg) was dialyzed against 10 mM potassium aspartate, 10 mM sodium borate, and 10 mM imidazole-HCl, pH 7.0, to reduce the conductivity below 1.0 mmho. The sample was made 10% in sucrose and placed on top of the upper gel in a Canalco preparative electrophoresis...
Fig. 1. Elution profiles of glutaminase from ion exchange and gel filtration columns. A, DE23 cellulose chromatography. The column (13.5 x 50 cm) was equilibrated with buffer, loaded with sample, and a gradient developed as described under Step IV of "Purification." The sample volume was 5900 ml containing 50 mg/ml of protein. Fractions of 100 ml were collected. Flow rate was 35 ml/cm²/hour at room temperature. B, gel filtration with 8% agarose. Two tandem columns (5 x 120 cm) of Bio-Gel 1.5m were equilibrated with standard buffer containing 10 mM imidazole-HCl, 20 mM potassium aspartate, and 20 mM sodium borate at pH 7.1. The sample volume was 200 ml containing 50 mg/ml of protein. Nineteen milliliter fractions were collected. Flow rate was 10 ml/cm²/hour at 4°. C, DE52 cellulose chromatography. The column (10 x 30 cm) was equilibrated, loaded, washed, and developed as described under Step VI of "Purification." The sample volume was 3700 ml containing 1.3 mg/ml of protein. Fractions of 19 ml were collected. Enzyme activity (△) was determined using the standard assay system ("Methods"). Absorbance at 280 nm (△) was used as a measure of protein concentration and conductivity (○) of each fraction was determined as a measure of total salt concentration.

A summary of the purification in Table II shows that glutaminase B activity was enriched more than 6,000-fold by the procedures described above with an overall yield of 19%. The greatest loss of enzyme occurred in Step IV. This loss was prevented by more rapid adsorption and elution on a smaller scale.

Purity of Enzyme—Analytical polyacrylamide gel electrophoresis was used to assess the purity of the 6,000-fold enriched glutaminase B preparation. Shown in Fig. 3 are duplicate gels which were run at pH 7.8 (System D of Rodbard and Chrambach (14)) and stained for glutaminase activity with nitroblue tetrazolium or for protein with Coomassie blue. An equal amount of protein (100 μg) was applied to each gel. A protein band corresponding to glutaminase B activity could not be observed in the sample which was 450-fold purified (Step VI). A protein band which migrates with glutaminase B activity is detected with enzyme purified by electrophoresis. This protein band comprised at most 40% of the total protein as determined by spectrophotometric scanning at 540 nm.

Similar results were obtained when electrophoresis in polyacrylamide gels at pH 9.6 (system of Ornstein and Davis (15)) was performed and when electrophoresis on cellulose acetate strips at pH 7 and pH 8.5 with imidazole and barbital buffers, respectively, was carried out.

Isoelectric focusing in polyacrylamide gel slabs also showed...
FIG. 2. Elution profile of *Escherichia coli* glutaminase B activity from 10% polyacrylamide gel electrophoresis column (2.6 x 3.75 cm). The gel column was prepared as described under "Methods" and run as described under Step VII of "Purification." The sample volume was 5 ml containing 15 mg/ml of protein. Fractions of 10 ml were collected at 4°C. Absorbance at 540 nm (D — D) was used to determine the position of the tracking dye. Absorbance at 280 nm (Δ — Δ) was used as a measure of protein concentration, and enzyme activity (A — A) was determined using standard assay conditions.

FIG. 3. Polyacrylamide gel electrophoresis performed at pH 7.2 (System D). Gels were stained for glutaminase activity (A and C) and protein (B and D) as described under "Methods." A and B, 100 μg of purified protein from Step VI of "Purification" was applied to each gel; after electrophoresis the gels were stained. C and D, 100 μg of purified protein from Step VII of "Purification" which used preparative electrophoresis; 7.5% acrylamide gels were used. Conditions for electrophoresis are described under "Methods.

that the protein band corresponding with glutaminase B activity comprised approximately 40% of the total protein of the 6,000-fold purified preparation.

Properties

Kinetic Parameters—The pH dependence of glutaminase B is depicted in Fig. 4. The enzyme has a broad pH optimum between 7.1 and 9.0. At pH 5 which is the optimum for glutaminase A, glutaminase B had less than 5% of its maximum activity. The curves for potassium phosphate or potassium phosphate plus sodium acetate and glycine were identical as shown. The formation of γ-glutamyl hydroxamate from l-glutamine and NH₂OH had a more basic pH optimum from 8.3 to 9.2. The rate of the hydrolytic deamidation is approximately 100 times that of the maximum velocity of the γ-glutamyl hydroxamate formation under the conditions of assay. Although glutaminase B has a broad pH optimum above 7, the enzyme was more stable at pH 7 than at pH 8 or 9 in the absence of stabilizing ligands. This pH-dependent difference in stability was not observed in the presence of aspartate or borate.

Glutaminase B exhibits complex substrate saturation kinetics for l-glutamine as illustrated in Fig. 5. The curve has an intermediary plateau region from 8 to 13 mM l-glutamine. The intermediary plateau region was observed in all preparations of glutaminase B from crude extracts to the 6,000-fold purified enzyme. The plateau could be shifted to lower levels of substrate by preliminary incubation of the enzyme with dicarboxylic acids, or by heating at 23°C. The plateau was shifted to higher levels of substrate by preliminary incubation with ATP. The same intermediary plateau region was observed when the assays were performed with potassium phosphate or imidazole-HCl buffer, both pH 7.1. None of the conditions which were examined, resulted in elimination of the intermedi-
Glutaminase B has a high degree of substrate specificity. The enzyme catalyzed only the deamidation of L-glutamine or the formation of γ-glutamyl hydroxamate from L-glutamine and NH₄OH was measured. Glutaminase B did not hydrolyze D-glutamine, L-asparagine, α-asparagine, or isoglutamine. In addition, L-glutamate could not substitute for L-glutamine in the formation of γ-glutamyl hydroxamate. The high degree of substrate specificity exhibited by glutaminase B is similar to that observed with glutaminase A (3) but in contrast to several other bacterial glutaminases which also deamidate asparagine (23).

Isoelectric Point—The isoelectric point of glutaminase B determined by isoelectric focusing on ampholytes in polyacrylamide gel was 5.4 using a pH 3 to 10 carrier (Fig. 6). Consistent with an isoelectric point of 5.4 is the observation that glutaminase B precipitates at pH 5. In contrast, glutaminase A remains soluble at pH 5 as expected (4).

Molecular Weight—The molecular weight of glutaminase B was estimated by gel filtration and polyacrylamide gel electrophoresis (Fig. 7). Chromatography of glutaminase B on Sephadex G-200 gave a molecular weight of approximately 90,000. Analytical polyacrylamide gel electrophoresis as pH 7.8 also gave a molecular weight of approximately 90,000 for glutaminase B while gel electrophoresis at pH 9.6 consistently gave a slightly higher molecular weight of about 100,000.

Cold Lability

Effect of Temperature on Time-dependent Activation and Inactivation—Exposure of glutaminase B to cold resulted in a reversible inactivation of enzymatic activity, while subsequent warming restored the activity. After partial purification of glutaminase B at 4° in the absence of stabilizing ligands, the enzyme exhibited a low level of activity. Warming the enzyme at 24° resulted in a 2- to 5-fold increase in activity. Maximum activation required approximately 35 min even though the temperature of the enzyme solution reached 24° after only 20 s. Upon rapidly cooling the warm-activated enzyme to 4° within 20 s, a slow decrease in the catalytic activity was observed. Approximately 45 min were required for the enzymatic activity to return to the control level. Both the activation by warming and the inactivation by cooling were first order processes with respect to time. The t₅₀ for activation was 7.7 min while the t₅₀ for inactivation was 28 min (Fig. 8B).

Activation by warming and inactivation by cooling of glutaminase B at various temperatures below 24° also exhibited first order kinetics. These results are summarized in Table III. Between 8.5° and 24.0°, the warming temperature was inversely proportional to the t₅₀ for activation. The t₅₀ for activation at 8.5° was 16.9 min, at 16.5° it was 12.0 min, and at 24° it was 7.7 min. The t₅₀ for inactivation by cooling from 24° was 28 min, and the t₅₀ from 16.5° increased to 51 min. Since the half-time for inactivation at 4° is almost double when cooling from 16.5° compared to cooling from 24°, the inactivation processes appear to depend upon the starting temperature and are at least partially different in these two cases.

In Fig. 9, the extent of activation by warming the enzyme at various temperatures for 60 min is plotted. The activation increases as the warming temperature is increased up to 24°. When the warming temperature is increased above 24° the activation decreases. No activation occurred at 37°.

Reversible cold inactivation of glutaminase B was not only observed in the partially purified enzyme preparations as described above, but it was also seen in highly purified preparations enriched 6,000 fold for glutaminase B activity. In addition, varying the protein concentration of the preparation from 0.1 to 50 mg/ml did not effect the activation by warming or inactivation by cold.
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Fig. 8. Time course for activation and inactivation of glutaminase B by warming and cooling. Aliquots were assayed for activity under standard assay conditions at various times as indicated on the abscissa. A, increase in glutaminase B activity with preincubation at 24°C (O—O). Decrease in glutaminase B activity upon cooling at 4°C (•—•). B, log plots of the activity of glutaminase upon heating and cooling. Velocity (V) is specific activity (units/mg). One unit of enzyme is the amount that catalyzes conversion of 1.0 μM of substrate to product per min under standard assay conditions.

TABLE III

Preincubation of glutaminase B at various temperatures

<table>
<thead>
<tr>
<th>Activation temperature</th>
<th>t₁/₂ for activation</th>
<th>t₁/₂ for inactivation</th>
</tr>
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<tbody>
<tr>
<td>24°C</td>
<td>7.7</td>
<td>28</td>
</tr>
<tr>
<td>20</td>
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</tr>
<tr>
<td>16.5</td>
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<td>11.5</td>
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</tr>
<tr>
<td>8.5</td>
<td>16.0</td>
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</table>

* Aliquots of glutaminase B incubated at 4°C were activated by preincubation at the temperatures indicated.

The t₁/₂ for activation was calculated from plots of log₁₀ (maximum velocity - observed velocity) versus time at the designated temperature while the t₁/₂ for inactivation was calculated from plots of log₁₀ observed velocity versus time at the designated temperature.

* After maximal activation had occurred at the temperature indicated the enzyme preparation was cooled to 4°C and the inactivation process measured.

Fig. 9. Activation of glutaminase B by warming at different temperatures. Aliquots were warmed for 60 min and then assayed under standard conditions. Vₐ is velocity of enzyme reaction after warming; V₀ is velocity of unwarmed sample (4°C).

An Arrhenius plot of the activation process is shown in Fig. 10. Glutaminase B was warmed at various temperatures between 4°C and 24°C and the activity determined as a function of the warming time similar to the experiment shown in Fig. 8. From the first order activation plots, the slopes were calculated and then plotted as a log function of the reciprocal of the absolute warming temperature. A linear function was described below 20°C and the activation energy of the activation process was calculated to be 5,900 cal/mol. Although activation of the enzyme may involve several processes, each of which has a different activation energy, the linear Arrhenius plot shown in Fig. 8 permits a crude estimation of the overall activation process between 8°C and 20°C. Indeed, the activation process is probably quite complicated, since half-times for inactivation at 4°C are dependent upon the temperature at which the enzyme was previously activated. The break in the Arrhenius plot above 20°C is consistent with the data presented in Fig. 2, showing that warming the enzyme at temperatures above 24°C results in a decreased activation.

Effect of Temperature on Substrate Saturation Kinetics—Activation of glutaminase B by warming was found to alter the Vₘₐₓ, Sₘₐₓ, and shape of the glutamine substrate saturation plot. The substrate saturation plot shows complex kinetics with an intermediary plateau region (Fig. 11) (24). At saturating levels of substrate the maximum velocity (Vₘₐₓ) was increased 2-fold by warming at 23°C. The activation by warming at 23°C also reduced the concentration of L-glutamine required for half-maximal velocity (Sₘₐₓ) from 5 to 2.5 mm. In addition,
the intermediary plateau region was shifted to slightly lower concentrations of glutamine and made more pronounced by the warming process. These changes in the substrate saturation plot effected by warming at 23° were similar to those observed after preliminary incubation of the enzyme with L-glutamate at 4° (25). Intermediary plateau regions in substrate saturation plots have been reported for several other enzymes (26).

Effect of Temperature on Molecular Size—Since many cold-labile enzymes have been shown to undergo alterations of molecular weight upon activation by warming, the molecular weight of warm activated and cold inactivated glutaminase B was estimated. To minimize the time required for molecular weight determination, polyacrylamide gel electrophoresis was used (18) and the temperature monitored during electrophoresis using a thermistor probe embedded in a gel. After electrophoresis the gels were stained for glutaminase B activity. No difference in the electrical charge of the cold-inactivated and warm-activated enzymes on assay temperatures was broken. The activation energy calculated from the slope of the curve for activity versus time was constant for the first 5 min, but afterwards the slope increased. This increase in slope indicates that some activation did occur during the assay.

Stability—Initial attempts to purify glutaminase B were hampered by the cold lability of the enzyme and its lack of long term stability at room temperature. Of the many ligands tested, sodium borate at 4° was the most effective stabilizing agent. Five-fold purified preparations of glutaminase B lost no activity after 13 days in the presence of sodium borate while the 6,000-fold purified enzyme was completely stable for 5 days. L-Glutamate and L-aspartate were less effective stabilizing agents than borate while L-glutamine, phosphate, and chloroacetamide, diisopropyl phosphorofluoridate, and phenylmethanesulfonyl fluoride, Glutaminase B in both crude and purified preparations lost significant activity upon freezing at 20° and 80°, in the presence and absence of 20% glycerol. In order to stabilize the enzyme, both aspartate and borate were included in all buffers used throughout the purification.

DISCUSSION

Glutaminases are ubiquitous in organisms but their properties vary. E. coli has two glutaminases, A and B, with different properties. Glutaminase B appears to be a constitutive enzyme since its level could not be increased by altering the nutritional conditions of the culture medium. In contrast, the levels of glutaminase A are under rigorous control by cyclic AMP and nitrogenous metabolites (22). From the purification scheme it is apparent that glutaminase B comprises less than 0.01% of the total cellular protein. Thus, glutaminase B is a microconstitutive enzyme like nicotinamide deaminase also from E. coli (27). In the case of nicotinamide deaminase, mutants which overproduce the enzyme were found and the task of enzyme characterization was considerably facilitated. Attempts to isolate mutants that hyperproduce glutaminase B have been unsuccessful.
The physical properties of glutaminase B are not dissimilar to those recorded for other glutaminases. A molecular weight of approximately 90,000 for glutaminase B is similar to that for glutaminase A (110,000) from Escherichia coli (3), glutaminase (110,000) from Clostridium welchii (28) while molecular weight determinations for glutaminase-asparaginase from pseudomonas range from 25,000 to 137,000. The molecular weights of glutaminases from mammalian kidney, brain, and liver of pig (29, 30) and rat (31) range from 95,000 to 140,000. The enzymes from pig have been shown to polymerize into aggregates of 2,000,000 molecular weight. No polymerization of glutaminase A was observed under a variety of conditions.

Glutaminase B exhibits complex substrate saturation kinetics. The plot for L-glutamine saturation shows an intermediary plateau region which may be shifted by preliminary incubation of the enzyme with various effectors. These plateau regions have been observed with several other enzymes: CTP synthetase, (32), phosphoenolpyruvate carboxylase (33), and threonine deaminase, both all from Escherichia coli; glutamate dehydrogenase from Blastocadium emersonii (34); and DT diaphorase (35) and β-OH butyrate dehydrogenase* from rat liver and brain, respectively. In almost all cases the plateau region may be altered or even eliminated by the addition of specific ligands. In the case of glutaminase B the plateau may be shifted to the left and narrowed by preliminary incubation with L-glutamate or by prior exposure to 23°C. The plateau observed with DT diaphorase may be completely eliminated by Tween or phosphatidyicholine (35). Although kinetic analyses have suggested some common mechanistic features for the generation of intermediary plateau regions (39), the examples cited above indicate that the precise mechanisms probably vary widely.

Glutaminase B is active at neutral and alkaline pH like most of the glutaminases from microorganisms and mammals that have been examined. Glutaminase A from Escherichia coli (3) and glutaminases from Clostridium welchii (28) and Proteus morganii (36) are exceptions since these enzymes have pH optima at 5.

Enzymes like glutaminase B which exhibit a loss of catalytic activity upon exposure to cold temperatures above freezing are said to exhibit cold lability, cold inactivation, or cold sensitivity. This phenomenon has been reported for enzymes from many species of animals, plants, and microorganisms (24, 37-72). Cold-labile enzymes are not confined to one specific type of enzymatic reaction, to a single metabolic pathway, or to groups of enzymes with similar functions. To date no firm evidence exists which implicates cold lability as a regulatory mechanism in metabolism; but it has been suggested that cold lability may participate in controlling the metabolism in hibernators (73).

In many instances cold lability is conferred or accelerated by a particular ligand while another ligand can protect against cold inactivation. There is no absolute correlation with the effect of the ligand on enzyme activity and its ability to influence cold inactivation. Often activators or substrates prevent cold inactivation as in the case of glutaminase B (4), 17β-hydroxy steroid dehydrogenase (50), glycogen phosphorylase (46), and pyruvate carboxylase (47). However, there are examples where activators or substrates accelerate cold inactivation as noted with pyruvate kinase (48) and pyruvate carboxylase (47), respectively.

Almost all known cold-labile enzymes are multimeric proteins. Cold inactivation may cause the dissociation of the enzyme into inactive dimers as in the case of argininosuccinase (45), glyceraldehyde-3-P dehydrogenase from rabbit muscle (53), threonine deaminase (66), and tryptophanase (39, 40), glucose-6-P dehydrogenase (49), glutamic dehydrogenase (42, 43), and glyceraldehyde-3-P dehydrogenase from yeast (53). Glycogen phosphorylase from rabbit muscle (46) and lactic streptococcus proteinase (56) have both been found to aggregate upon inactivation by cold while ribulose-diphosphate carboxylase from tobacco shows no change in sedimentation after cold inactivation (68). Because glutaminase B is a microconstitutive enzyme, pure enzyme was not available to search for catalytically inactive species of altered molecular weight. Gel filtration and polyacrylamide electrophoresis on partially purified preparations of glutaminase B did not reveal any detectable change in the molecular weight of active species.

The precise molecular mechanism of cold lability remains to be established, but several investigators have suggested that conformational changes may occur upon exposure of these enzymes to cold (74-76). These changes in conformation probably precede the observed alterations in molecular weight. Modulation of cold lability by specific ligands, salts and pH is consistent with the above hypothesis. Fructose diphosphate which is an allosteric activator increasing the cold lability of pyruvate kinase (48), and borate protecting against cold inactivation of glutaminase B, are two of many examples where the interaction of a specific ligand with an enzyme leads to an increase or decrease in cold lability of that enzyme. Presumably the above ligand-enzyme interactions produce conformational states which show increased or reduced stability toward further alterations by cold. Conformational changes which do occur upon exposure to cold, probably result from a weakening of the interactions among hydrophobic groups in the protein (74-76). The weakening of the association among apolar groups is enhanced at low temperatures as water molecules assume a more structured array. An activation energy of 5,900 cal/mol reported above for glutaminase B activation by warming is consistent with the suggestion that exposure to cold produces conformation changes that lead to an inactivation of catalytic activity.

Acknowledgments—We wish to express our appreciation to Doctors Ann Ginsburg and Theresa Stadtman for many helpful discussions and to Henry Lutterlough, Maurice Miles, and David Rogerson for the large scale growth of Escherichia coli. We also thank the New England Enzyme Center for help in the large scale purification of glutaminase B.

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