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A Binding Protein for Glutamine and Its Relation to Active Transport in *Escherichia coli**

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SUMMARY

Escherichia coli contains a binding protein for glutamine which has been isolated by osmotic shock, purified, and characterized. It has a K_D of 3×10^{-7} M and, of the naturally occurring amino acids, only glutamine is bound. The γ-glutamylhydrazide and γ-glutamylhydroxamate competitively inhibit both the binding reaction and the uptake of glutamine by intact cells. The protein exhibits native tryptophan fluorescence with an emission maximum at 336 nm when excited at 280 nm. Addition of 1 μM glutamine specifically causes a spectral shift and quenching of fluorescence. The kinetics of binding have been investigated by following the fluorescence change using a stopped flow apparatus. The k for the forward reaction is 9.8×10^7 M^{-1} sec^{-1} and the k for the back reaction is 16 sec^{-1}.

The transport of glutamine is also highly specific, with a K_m of 0.8×10^{-7} M. A mutant has been isolated which has a 3-fold higher initial rate of transport and 3.0 times more binding protein than the parent strain. Other mutants, resistant to γ-glutamylhydrazide, have been isolated which have only 10% of the initial rate of transport and about 10% of the binding protein. These data suggest a role for the glutamine binding protein in active transport. Furthermore, growth in a rich medium represses both transport and the formation of binding protein. Additional evidence is provided by the fact that the initial rate of glutamine uptake is reduced 90% by osmotic shock while certain other transport systems are maintained, and this is associated with release of the glutamine binding protein.

It has previously been shown that *Escherichia coli* accumulates amino acids by systems which are specific for individual amino acids and by systems which are general for groups of amino acids (1–5). For example, on the basis of kinetic evidence, transport systems have been described that are specific for leucine (6) on the one hand, and specific for leucine, isoleucine, and valine on the other (7). Associated with the presence of these transport systems is the occurrence of shock-releasable binding proteins of similar specificities. In the present communication we are concerned with the highly specific uptake system for glutamine; no other natural amino acid competes for active transport. When our strain of *E. coli* was subjected to osmotic shock, a binding protein was released, also highly specific for glutamine. The protein has been purified to homogeneity and its properties are described. The kinetic data of the binding reaction have been measured using stopped flow methods. Examination of mutants and other studies are described which lead us to believe that the glutamine binding protein is involved in active transport.

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MATERIALS AND METHODS

Bacteria and Media—*E. coli* strain 7 (8), a derivative of K-12 Hfr Cavalli, was the gift of Dr. E. C. C. Lin. A mutant which could grow on glutamine as sole carbon source, GLNP 1 (9), was derived from strain 7 by mutagenesis, using *N*-methyl-*N*-nitro-*N'*-nitroso guanidine (10). Strain GLNP 1 was used in all experiments except as noted. Strain GH 20 was selected from GLNP 1 by its resistance to 5×10^{-4} M γ-glutamylhydrazide. Unless otherwise stated, all cultures were grown in a synthetic minimal medium described by Tanaka, Lerner, and Lin (11), supplemented with 1% sodium succinate (Baker and Adamson, Morristown, New Jersey). For studies of repression of synthesis of the glutamine binding protein associated with repression of transport, a complex medium containing 3% dehydrated Tryptone and 4% yeast extract was used (both from Fisher Scientific). Bacterial cultures were maintained on nutrient agar slants that were transferred monthly. The mutants were single colony isolates and, like the parent strain, were sensitive to f2 phage and were alkaline phosphatase negative.

Chemicals—For most studies L-[U-^{14}C]glutamine (212 mCi per mmole) was diluted 10-fold with nonradioactive glutamine. The isotopic material was obtained from New England Nuclear, and nonradioactive L-amino acids from Mann Research. The γ-glutamylhydrazide was purchased from Nutritional Biochemicals, and chloramphenicol from Sigma. The γ-glutamylhydroxamate (L-glutamic acid-γ-monohydroxamate) was the gift of Dr. A. L. Neal. Bio-Gel P-10 was from Bio-Rad Laboratories, Richmond, California, and Whatman DEAE-cellulose DE-52 from H. Reeve Angel and Company, Clifton, New Jersey.

Transport Assays—The temperature was maintained at 23° throughout the following procedure. Cells were harvested by centrifugation, twice washed with minimal medium, and suspended in the same medium (1 g, wet weight, per 40 ml). For

transport measurements a fraction was incubated for 5 min in the presence of 10 mM glucose and 80 μg per ml of chloramphenicol. A portion of this suspension (usually 30 μl) was added to the final reaction mixture so that 0.5 ml contained 10 mM glucose, 6 μM labeled glutamine, and 80 μg per ml of chloramphenicol, in minimal medium. After 15 and 30 sec, a 0.2-ml portion was filtered on a 25-mm nitrocellulose filter (type HA, 0.45 μ, Millipore Corporation, Bedford, Massachusetts), and washed with 10 ml of 0.01 M Tris-HCl, pH 7.3-0.15 M NaCl-5 × 10^{-4} M $MgCl_2$. This wash medium has been shown to give consistently good results, with very low blank values for poisoned cells, and the retention of transported amino acid was often greater than when cells were washed with growth medium. In all experiments, including determination of K_m values, the concentration of bacterial cells was adjusted so that less than 10% of the substrate was taken up. The filters were dried and counted in a Nuclear Chicago Unilux II liquid scintillation counter, using a solution of 15 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene dissolved in 3.81 liters of toluene.

Binding Assays—For quantitative work, equilibrium dialysis was carried out in Plexiglass chambers, as previously described (6). Side A contained protein, 0.01 M potassium phosphate, pH 7.0, 0.05 M NaCl, and a near saturating concentration of chloroform in a total volume of 0.1 ml. Side B contained isotope, 0.01 M potassium phosphate and 0.05 M NaCl in 0.1 ml. The concentration of glutamine (20 mCi per mmole) was 10 μM unless otherwise stated. The chambers were rotated overnight at 2° after which 0.05-ml samples were removed from each side and counted by liquid scintillation in a solution consisting of 15 g of 2,5-diphenyloxazole, 0.188 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, 1 liter of Triton X-100, and 2 liters of toluene. To assay column fractions a filter binding assay was used (12). A final volume of 0.2 ml contained protein, 0.05 ml of 2 × 10^{-5} M glutamine, and water. After addition of 0.05 ml of 1 M $MgCl_2$, 0.2-ml portions were filtered on 25-mm B-6 filters (Schleicher and Schuell, Keene, New Hampshire) washed with 0.5 ml of 0.2 M $MgCl_2$ at room temperature, dried, and counted as in the transport assays. One unit corresponds to 1 nmole of glutamine bound and specific activity is expressed as units per mg of protein.

Osmotic Shock—A modification of the procedure of Neu and Heppel (13) was used. For small scale, analytical studies, cells in midexponential stage were twice washed with cold Tris-HCl, 0.01 M, pH 7.3, containing 0.03 M NaCl. The cells were then suspended in 40 volumes (w/v) of 0.033 M Tris-HCl, pH 7.3, containing 20% sucrose and 0.2 mM EDTA, at 23°. The suspension was swirled for 5 min and centrifuged. The pellet was rapidly resuspended in 40 volumes of cold 0.5 mM $MgCl_2$ and swirled for 10 min. The suspension was centrifuged at 2°, the shock fluid was removed, and the pellet of shocked cells was resuspended in 0.033 M Tris-HCl, pH 7.3, at 23°. The supernatant (crude shock fluid) was concentrated by ultrafiltration in an Amicon model 401 ultrafiltration cell equipped with a UM-10 membrane (Amicon Corporation, Lexington, Massachusetts).

For preparation of binding protein on a large scale, cells were grown in 15-liter carboys and aerated with filtered compressed air through glass spargers. Cells in late stationary phase were collected in a Sharples refrigerated centrifuge. The cells were twice washed in 20 volumes of cold 0.01 M Tris-HCl, pH 7.2, 0.03 M NaCl. The packed cells were suspended in 15 volumes of 0.033 M Tris-HCl, pH 7, 23°, in a Waring Blendor; to this was added an equal volume of 0.033 M Tris containing 40% sucrose and 4 mM EDTA. The suspension was swirled for 5 min and centrifuged. The pellet of cells was rapidly dispersed in 30 volumes of cold distilled water. After 3 min, $MgCl_2$ was added to a concentration of 1 mM, and the suspension was swirled for 10 min. Shock fluid was obtained by centrifugation at 2° and concentrated as before.

Preparation of Antisera—New Zealand white rabbits were inoculated with 1 mg of pure glutamine binding protein in Freund's adjuvant. After 30 days they received booster injections of 0.5 mg of protein by intravenous injection. Ten days later the rabbits were bled and the antisera obtained. Antibody titer was measured by serial dilutions in microcapillary tubes. Protein was determined by a modification of the procedure of Lowry *et al.* (14) with bovine serum albumin as standard.

TABLE I

Purification of glutamine binding protein

Fraction	Total protein	Total units	Total volume	Specific activity	Recovery
	mg	*units*	*ml*	*units/mg protein*	%
Crude shock fluid	1900	6800	275	3.5	
DEAE-cellulose	300	6400	25	22	95
Electrofocusing	180[a]	5400	7	34	85

[a] This step was actually carried out three times on 100-mg portions of the DEAE-cellulose fraction. The electrofocusing elution pattern has been published (9).

RESULTS

Glutamine Binding Protein

Purification of Glutamine Binding Protein—Our previous method (9) has been modified in order to increase the yield; it is now possible to isolate about 200 mg of pure binding protein within a week. A mutant, GLNP 1, was used because it has elevated levels of transport and binding protein. No differences could be found between protein derived from mutant and parent strain 7, or in the results obtained with cells in stationary and exponential phase. The bacteria were grown to late stationary phase in order to increase the yield.

Four hundred grams (wet weight) of *E. coli* GLNP 1 were osmotically shocked and the crude shock fluid (12 liters) was concentrated by ultrafiltration using a 6-inch Amicon UM-10 membrane. The concentrate (150 ml) was chromatographed on a Bio-Gel P-10 column of 1 liter capacity, equilibrated with distilled water at 4°, to remove small molecules. The first protein peak to emerge (1800 mg) was concentrated to 300 ml by ultrafiltration and stored at −90°. Material at this stage had a specific activity of 3.5 units per mg (Table I).

DEAE-cellulose Chromatography—A 200-ml column (2.5 × 41 cm) of Whatman DEAE-cellulose DE-52 (microgranular, 1 meq per g) was equilibrated at 4° with 5 mM Tris-HCl, pH 7.4. The concentrated Bio-Gel fraction was applied and the column was washed with 2 column volumes of 5 mM Tris-HCl, pH 7.4. The glutamine binding protein does not adsorb at this pH because of its basic character and it appeared in the flow-through fraction. Absorbance at 280 nm was monitored by means of a Uvicord II flow cell (LKB Products, Rockville, Maryland).

Fractions containing glutamine binding protein by the filter binding assay were pooled and concentrated to yield 300 mg of protein with a specific activity of 22 units per mg of protein.

Isoelectric Focusing—The concentrated material from the DEAE-cellulose column was divided into three equal parts. Each portion (100 mg) was electrofocused in an LKB model 8102 apparatus with a 440-ml column containing 1% ampholytes in a sucrose gradient (0 to 50%, w/v) and with a pH gradient of 7 to 9. Sulfuric acid (1%) was used for the lower positive electrode and ethanolamine (1%) for the upper negative electrode. The ampholytes were focused for 24 hours at 1000 volts after which a sample of ampholytes and sucrose was removed from about 3 inches below the gradient-ethanolamine interface, evaporated to near dryness, and combined with the protein solution. This material, of about the original volume, was reapplied to the column where it was isopycnic. The voltage was applied for another 24 hours. The column was emptied by allowing the pH gradient to flow through an LKB Uvicord II flow cell spectrophotometer, which made it possible to follow the optical density at 280 nm. Fractions of 5 ml each were collected; their pH and binding capacity were measured. The binding protein appeared in the gradient at pH 8.6. Fractions containing glutamine binding activity were dialyzed against two changes of 0.1 M NaCl and then against three changes of water; 186 mg of specific activity 34 units per mg were recovered in 85% yield.

Tests of Purity—The protein obtained from the isoelectric focusing step was judged to be homogeneous by the following four criteria. (*a*) The protein migrated as a single band in polyacrylamide disc gel electrophoresis at pH 9.5 in the Tris-glycine system of Ornstein and Davis (15) and in the pH 4.2 β-alanine system of Reisfeld (16) (Fig. 1). In addition, it migrated as a single band in gels of 6, 8, 10, and 12% acrylamide. (*b*) Antibody made to purified glutamine binding protein gave only one precipitin band in Ouchterlony double diffusion plates (18) using pure glutamine binding protein as antigen (Fig. 2). The antisera did not cross-react with the leucine-specific binding protein (6), the leucine-isoleucine-valine binding protein (7), the lysine-arginine-ornithine binding protein (5) and the cystine binding protein (19). (*c*) Sedimentation equilibrium centrifugation gave a straight line in a plot of concentration *versus* r (see Fig. 3). (*d*) No cysteic acid was found when a sample of protein oxidized with performic acid (20) was hydrolyzed and analyzed for its amino acid composition.

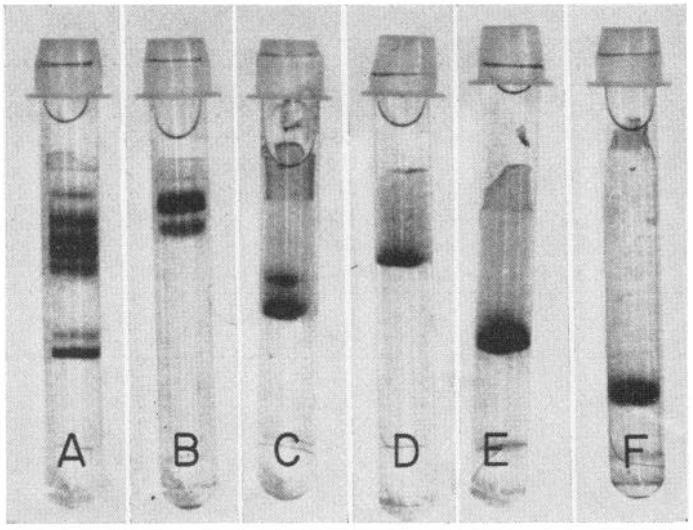


FIG. 1. Polyacrylamide disc gel electrophoresis. Disc gels were run as described in the text, at 4 ma per tube, at room temperature, for 30 min. Gels run at pH 9.5 were fixed in 12.5% trichloroacetic acid, stained with 0.05% Coomassie blue (17), and destained with 12.5% trichloroacetic acid. Gels run at pH 4.2 were fixed in 10% acetic acid, stained with 0.1% Amido black, and destained with 10% acetic acid. All gels were stored in 10% acetic acid to prevent shrinkage. *A*, crude shock fluid, pH 9.5, 10% gel; *B*, DEAE-cellulose fraction, pH 9.5, 10% gel; *C*, DEAE-cellulose fraction, pH 4.3, 10% gel; *D*, electrofocusing fraction, pH 4.3, 12% gel; *E*, electrofocusing fraction, pH 4.3, 8% gel; *F*, electrofocusing fraction, pH 4.3, 6% gel.

In order to show that the binding protein from GLNP 1 was identical with that from parent strain 7, three tests were applied. Antibody produced in rabbits against pure wild type protein gave one precipitin line with both proteins in Ouchterlony double diffusion plates. Both proteins were found to have a value for pI of 8.6, when isoelectric focusing was carried out. Finally, both proteins showed similar dissociation constants of approximately 0.3 μM.

Properties of Glutamine Binding Protein

Molecular Weight—The molecular weight was measured by sedimentation equilibrium in a Spinco model E analytical ultracentrifuge. A value of 24,000 was calculated from $d \log c/dr$ (Fig. 3), assuming a $\bar{v}$ of 0.73. The molecular weight was also determined by filtration on a column (1.5 × 33 cm) of Sephadex G-150. The glutamine protein and a number of standards were separately chromatographed with a blue dextran dye marker. The volume from the dye peak to the center of the protein peak was plotted *versus* log of the molecular weight (Fig. 4). A molecular weight of 29,000 was determined.

Amino Acid Composition—Purified glutamine binding protein (150 μg) was hydrolyzed in 6 N HCl at 110° in evacuated sealed

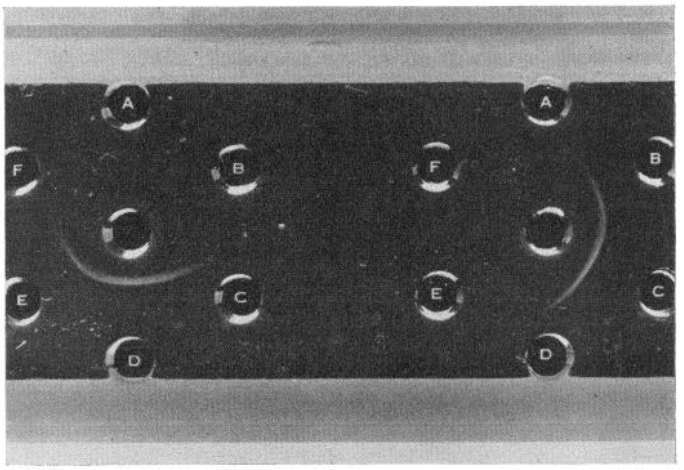


FIG. 2. Ouchterlony double diffusion precipitin test. Undiluted sera (10 μl, see "Methods") was placed in the center well of an 0.8% agarose plate. The plates were incubated for 24 hours at 23° in a closed humidity chamber. *Left*: *A*, leucine-isoleucine-valine binding protein, 2 μg; *B*, same, 10 μg; *C*, cystine binding protein, 10 μg; *D*, glutamine binding protein, 10 μg; *E*, glutamine binding protein, 2 μg; *F*, water. *Right*: *A*, arginine-specific binding protein, 10 μg; *B*, glutamine binding protein, 2 μg; *C*, glutamine binding protein, 10 μg; *D*, lysine-arginine-ornithine binding protein, 10 μg; *E*, leucine-specific binding protein, 10 μg; *F*, water.

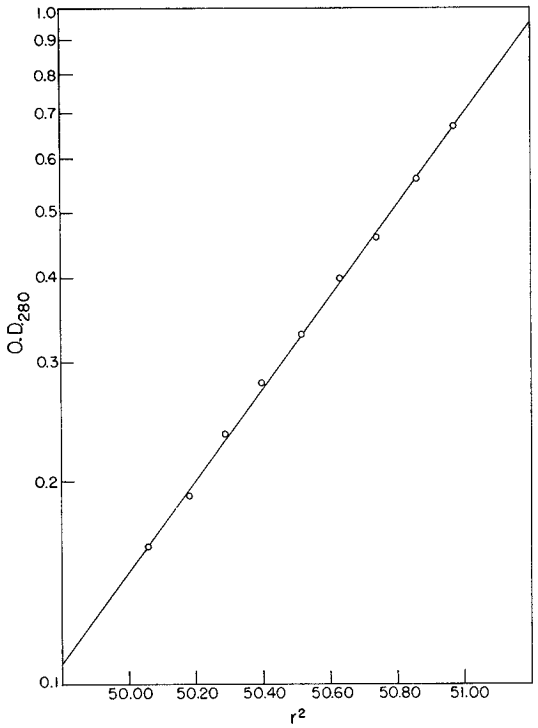


FIG. 3. Sedimentation equilibrium molecular weight determination. Glutamine binding protein was centrifuged at 34,000 rpm at 8° until equilibrium was reached, in a 12-mm double sector aluminum epoxy cell with sapphire windows. A 0.1-ml sample containing 0.05 mg of binding protein in 0.05 M potassium phosphate buffer, pH 7.0, was added to one sector and 0.1 ml of buffer to the reference side. The *abscissa* is measured from the center of the rotor. The *ordinate* is the optical density of the solution measured at 280 nm.

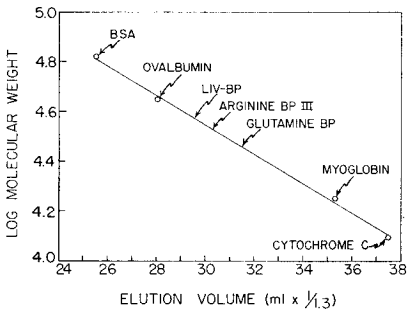


FIG. 4. Gel filtration molecular weight determination. A column (1.5 × 33) cm was filled with Sephadex G-150. The column was run at 30° and 1.3-ml fractions were collected. Each protein, about 0.1 mg, was separately chromatographed with a blue dextran dye marker. The elution volume is measured *from the center of the dye peak to the center of the protein peak. BSA*, bovine serum albumin; *LIV-BP*, leucine-isoleucine-valine binding protein.

tubes and subjected to amino acid analysis in a Beckman model 120C analyzer according to the method of Moore and Stein (21). The mean residues (Table II) were determined assuming 1 histidine per 26,000 molecular weight. It is interesting that the protein has no cysteine residues, in agreement with reports for other binding proteins. The large number of lysine and arginine residues account for its basic character.

Binding Properties—Of the natural amino acids only glutamine is bound (Table III) with a K_D of 3×10^{-7} M determined by

TABLE II

Amino acid analysis of glutamine binding protein

Pure glutamine binding protein (150 μg) was hydrolyzed in 6 N HCl in sealed evacuated tubes at 110° for 20 and 40 hours. The hydrolysate was evaporated to dryness and dissolved in 0.6 ml of water. Amino acid analysis was determined on a Beckman model 120 C machine. Assays were done in triplicate. Cysteine was determined by performic acid oxidation to cysteic acid followed by hydrolysis in 6 N HCl and amino acid analysis (20). The number of tryptophan residues were determined by two procedures: (*a*) *N*-bromosuccinimide oxidation and measurement of the change in absorbance at 280 nm (22), (*b*) measurement of the 288/280 ratio in 6 M guanidine-HCl according to the procedure of Edelhoch (23). Mean residues were determined by assuming 1 histidine per 26,000 molecular weight.

Amino acid	Hydrolysis for 20 hours (600 μg)	Hydrolysis for 40 hours (600 μg)	Mean residues
	μmoles		
Lysine	0.440	0.360	27
Histidine	0.016	0.014	1
Arginine	0.070	0.071	5
Aspartate	0.407	0.430	30
Threonine	0.154	0.120	9
Serine	0.092	0.094	6
Glutamate	0.217	0.210	15
Proline	0.081	0.082	6
Glycine	0.242	0.210	15
Alanine	0.298	0.280	20
Valine	0.181	0.190	13
Methionine	0.038	0.043	3
Isoleucine	0.139	0.140	9
Leucine	0.236	0.240	17
Tyrosine	0.118	0.120	8
Phenylalanine	0.132	0.130	9
Tryptophan			2
Cysteine			0

TABLE III

Competition for binding and transport of glutamine by other amino acids

Transport and binding assays were performed as outlined in "Methods." A concentration of 10 μM glutamine and 200 μM competing amino acid was used. Binding protein obtained by isoelectric focusing was used for all experiments in this paper.

Amino acid in addition to glutamine	Binding remaining	Transport
	%	%
None	100	100
Lysine	88	98
Arginine	85	100
Alanine	93	99
Asparagine	84	100
Aspartic	84	100
Glutamine	6	5
Leucine	89	99
Methionine	89	94
Phenylalanine	96	105
Isoleucine	89	104
Tryptophan	99	104
Serine	95	103
Hydroxy-L-proline	133	116
Valine	101	102
Glutamic acid	96	97

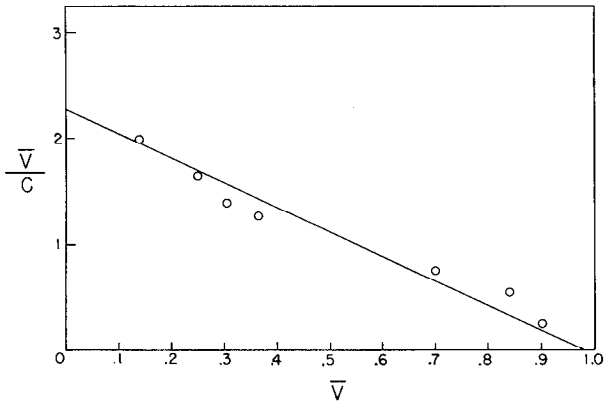


FIG. 5. Scatchard plot of glutamine binding protein. Binding assays were performed with 3 μg of glutamine binding protein and variable amounts of glutamine. $\bar{V}$, nmoles glutamine bound per nmole of protein. C, free concentration of glutamine (micromolar concentration).

TABLE IV

Competition for binding and transport by analogues of glutamine

Transport assays and binding assays were performed as outlined in methods. Analogues were used at a 20-fold excess over glutamine (10 μM glutamine and 200 μM analogue).

Analogue	Transport	Binding remaining
	%	%
None	100	100
Glutamic acid	97	96
Aspartic acid	100	84
Asparagine	100	84
γ-Glutamylhydroxamate	49	51
γ-Glutamylhydrazide	66	64
Azaserine	95	110
6-Diazo-5-oxo-L-norleucine	105	110
n-Acetyl glutamine	98	95

equilibrium dialysis (Fig. 5). The Scatchard plot (Fig. 5) indicates that 1 mole of glutamine is bound per mole of protein.

A number of analogues of glutamine have been assayed for inhibition of binding (Table IV). The limited survey suggests that alterations are tolerated only at the amide nitrogen; thus γ-glutamylhydrazide and γ-glutamylhydroxamate inhibit binding competitively (see below). The commonly used glutamine antagonist, 6-diazo-5-oxo-L-norleucine, is inactive.

The equilibrium level of glutamine binding is unaffected by pH over the range 3 to 9. It is also unaffected by alteration of ionic strength of the range 0.01 to 0.25 M. The labeled glutamine appeared to be unaltered after the binding reaction. It was chromatographically identical with a standard solution of glutamine by thin layer chromatography in butanol-acetic acid-water (4:1:5) and phenol-water (3:1).

Absence of Enzyme Activities—The protein was assayed for glutaminase activity according to the method of Meister (24) and for L-glutamine tRNA synthetase (25). Appropriate controls were run and 30 μg of protein were used per assay. No activity could be detected.

Stability—Like a number of other binding proteins (7, 26, 27) the glutamine binding protein is stable to storage at 3° and −90° for at least 6 months, and it is fully active after exposure

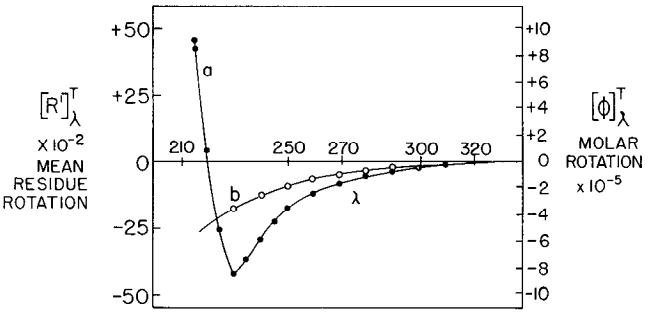


FIG. 6. Optical rotatory dispersion spectrum of glutamine binding protein. Spectra were run in a Jasco optical rotatory dispersion-ultraviolet model 5 instrument with a path length of 1 mm and a protein concentration of 80 μg per ml in water at 23°. A scale of 0.5 millidegrees was used. *a*, native protein; *b*, protein in 6 M guanidine hydrochloride.

to 100° for 10 min. It was reversibly denatured by 7 M urea and 6 M guanidine hydrochloride.

Optical Properties—The protein has a normal absorption spectrum with a 280:260 ratio of 1.65. From optical rotatory dispersion spectra (Fig. 6) it is clear that the α helical and β structure in going from native to denatured protein is drastically altered. The negative Cotton effect is abolished in guanidine hydrochloride. Plotting the long wave length data according to the method of Moffit and Yang (28) allows an estimate of helical content. The native protein yielded a $b_0 = -300°$; a value of zero was obtained for the protein in guanidine hydrochloride, suggesting a random coil. Assuming that a value of $b_0 = -630°$ corresponds to 100% helix (29), then the glutamine binding protein is approximately 45% α helix. No alteration in optical rotatory dispersion spectrum could be detected in the presence of 10 μM glutamine.

The protein exhibited native tryptophan fluorescence when excited at 280 nm. The emission maximum was at 336 nm in an Aminco-Bowman fluorometer. The emission wave length was calibrated using a mercury lamp to generate standard emission wave lengths. Tryptophan in solution has an emission maximum at 348 nm; thus the tryptophan in the glutamine binding protein is shifted to a lower wave length, indicating a more hydrophobic environment (higher energy) than for tryptophan in water. Addition of 1 μM glutamine caused the spectrum to shift towards the blue (higher energy), possibly indicating that tryptophans were now exposed to an even more hydrophobic environment. A drop in amplitude was also noted (Fig. 7). These changes were specific for glutamine. The closely related amino acids asparagine and glutamate caused no shifts (Fig. 7). The quenching of fluorescence at 340 nm could be titrated with glutamine. This yielded a $K_D = 3 \times 10^{-7}$ M, a value similar to the dissociation constant determined by equilibrium dialysis. The fluorescence was shown to be entirely due to the two tryptophan residues. Oxidation of the two tryptophans with *N*-bromosuccinimide (22) in 0.1 M acetate buffer at pH 4.5 completely destroyed the fluorescence at a molar ratio of 6 *N*-bromosuccinimide molecules per tryptophan. The oxidation of tryptophan residues caused by *N*-bromosuccinimide was prevented by glutamine (Fig. 8). However, as the destruction of tryptophan does not alter the binding activity, it is not possible to state whether tryptophan is at the active site and hidden by glutamine or whether glutamine causes a conformational change which buries the tryptophan residues.

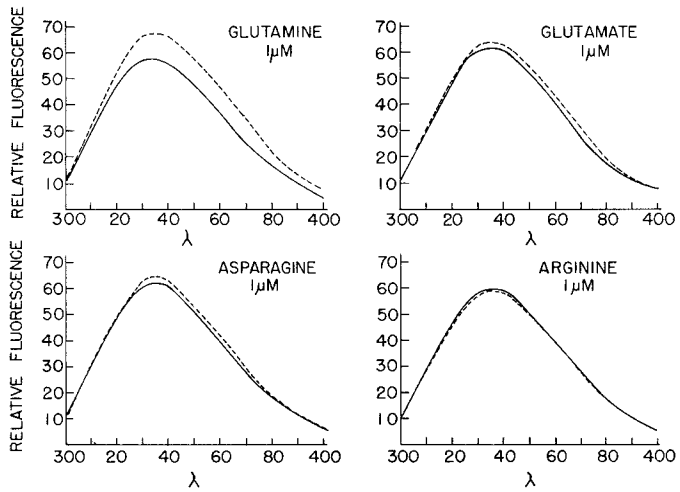


FIG. 7. Fluorescence of glutamine binding protein. A protein concentration of about 12 μg per ml in water at 23° was used. The protein was excited at 280 nm in an Aminco-Bowman spectrofluorometer using a 1-cm path length and appropriate entrance and exit slits. The fluorescence was determined as a function of wave length. - - -, relative fluorescence of the protein. ——, relative fluorescence when 1 μM of the appropriate amino acid was added. The amino acid was added in a small volume (0.1% volume change) and no correction was made for the volume change.

Kinetics of Binding—As mentioned above, addition of glutamine to the glutamine binding protein causes a quenching of fluorescence at 340 nm. With the use of a stopped flow apparatus (30) it is possible to measure the fluorescence change as a function of time and thus determine the rate constants of the binding reaction. Glutamine and glutamine binding protein at various concentrations were rapidly mixed in the stopped flow machine, the mixing chamber was excited at 284 nm and the fluorescence at 340 nm was measured. In Fig. 9 the rate of formation of the protein-glutamine complex is given for the following reaction, in which BP is binding protein and g is glutamine.

$$g + BP \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} (g)(BP)$$

$$k_D = \frac{k_{off}}{k_{on}}$$

$$\frac{d[(g)(BP)]}{dt} = k_{on}[(g)(BP)] - k_{off}[(g)(BP)]$$

A computer was used to find the best fit to the data by the method of least squares. The value of k_{on} was 9.8×10^7 M^{-1} sec^{-1}, S.D. = 0.82×10^7, and k_{off} was 16 sec^{-1}, S.D. = 1.7. This gives a K_D directly of 0.16 μM which is in good agreement with the K_D determined by equilibrium dialysis. The stopped flow measurements present special technical difficulty, combining an unusually high rate of reaction (1×10^8 M^{-1} sec^{-1}) with a rather small percentage change in fluorescence, and a requirement for excitation at 284 nm, where the output of the source is low. This combination of factors results in low precision and a poor signal to noise ratio which was only partly overcome by averaging 10 to 20 individual reaction records for each curve of Fig. 8.

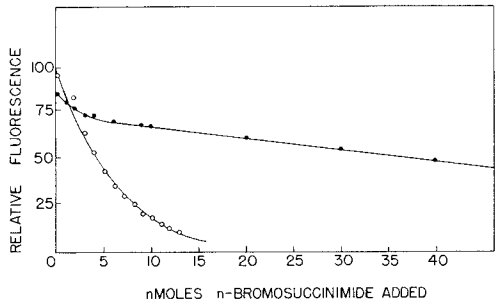


FIG. 8. Titration of tryptophan fluorescence by *N*-bromosuccinimide. Glutamine binding protein (3 nmoles) in 0.1 M acetate buffer, pH 4.5, was excited at 280 nm. Fluorescence was measured at 340 nm in an Aminco-Bowman spectrofluorometer, increasing amounts of *N*-bromosuccinimide were added. O——O, 3 nmoles of glutamine binding protein; ●——●; 3 nmoles of glutamine binding protein plus 100 nmoles of glutamine.

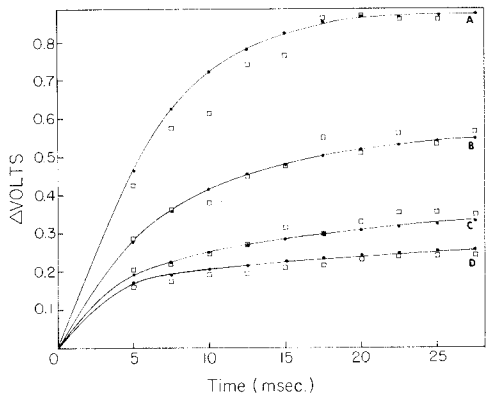


FIG. 9. Fluoroscence kinetics of the glutamine binding reaction. Glutamine (0.2 ml) and binding protein (0.2 ml) in 0.01 M potassium phosphate buffer at pH 7.2 were mixed. The *ordinate* is an arbitrary voltage scale obtained by estimating the total fluorescence change (and corresponding voltage change) at saturation for each amount of protein, and subtracting the voltage obtained at each time point after mixing, thus yielding a positive curve. The *abscissa* is time in milliseconds. □, experimentally determined points; ——, best computer fit to the data. The first point was taken 2½ msec after mixing and the lines were extrapolated to zero time. *A*, 2.5 μM glutamine and 2.5 μM glutamine binding protein; *B*, 1.25 μM glutamine and 1.25 μM glutamine binding protein; *C*, 0.625 μM glutamine and 0.625 μM glutamine binding protein; *D*, 0.625 μM glutamine and 0.312 μM glutamine binding protein.

Transport of Glutamine

General Properties—The K_m for transport of glutamine was 0.8×10^{-7} M both for strains 7 and GLNP 1 (Fig. 10), and the corresponding values for V_m were 10 and 30 nmoles per min per mg of protein (Table V). The uptake was linear for at least 3 min in strain 7 and for 1 min in GLNP 1 (Fig. 11). Of the analogues tested, only γ-glutamylhydrazide and γ-glutamylhydroxamate compete for glutamine transport (Table II). The γ-glutamylhydrazide showed a K_i of 75 μM for both transport and binding (Fig. 12, *a* and *b*).

Nature of Product of Transport—This was examined as follows. Cells were allowed to take up glutamine for 30 sec, washed on a Millipore nitrocellulose filter as in the transport assay, and then shocked with 10 ml of cold distilled water to release the small molecule pool. The extract was lyophilized and chromatographed on thin layer cellulose-gel plates, in butanol-acetic

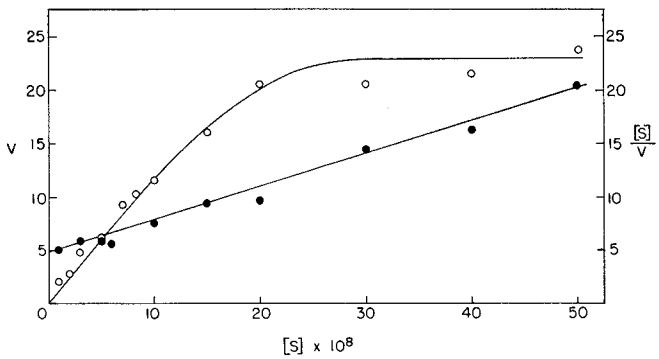


FIG. 10. K_m of glutamine uptake by strain GLNP 1. ○——○, initial rate of uptake, nanomoles per mg of protein per min (V). ●——●, $(S)/V$, molar per nmole per mg per min.

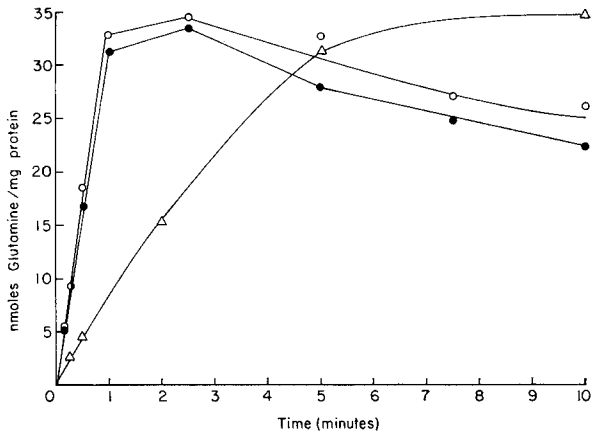


FIG. 11. Time course of uptake of glutamine by *E. coli* strains. △——△, parent strain 7; ●——●, GLNP 1; ○——○, GLNP 1 plus 0.6 mM azaserine. Cells were previously incubated at 23° for 15 min with and without azaserine. Uptakes were performed as outlined in "Methods."

TABLE V

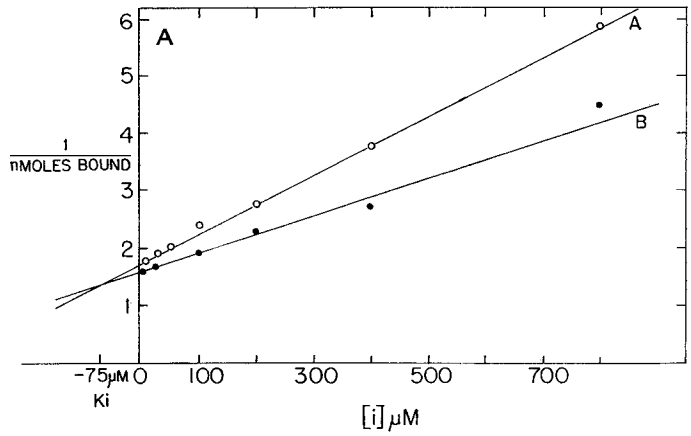
Comparison of the initial rate of glutamine uptake and amount of glutamine binding protein released by osmotic shock in E. coli 7 and in mutants derived from this strain

Transport assays, binding assays and composition of media are described in "Methods."

Strain	Medium	Initial rate of glutamine uptake	Glutamine binding protein released
		nmoles/mg/min	*units/g, wet weight, cells*
7	Minimal	10	7.4
7	Complex	3	2.0
GLNP 1	Minimal	34	18
GH 20	Minimal	1	0.8

acid-water (4:1:5). The product of transport was not glutamine, but rather chromatographed almost entirely with a marker of glutamic acid.

In spite of this conversion to glutamic acid, we believe that our kinetic transport data for glutamine are reliable, for the following reasons. (*a*) When the cells were previously incubated for 10 min at 23° in 0.6 mM azaserine, an inhibitor of γ-glutamyl transfer reactions, no significant change in initial (15 sec) rate of uptake was measured (Fig. 11). Under these conditions, 85% of the glutamine taken up remains as free glutamine. (*b*) Addition of a large excess of nonradioactive glutamate did not alter the initial kinetic parameters as would be expected if glutamine were first hydrolyzed and taken up as glutamate.



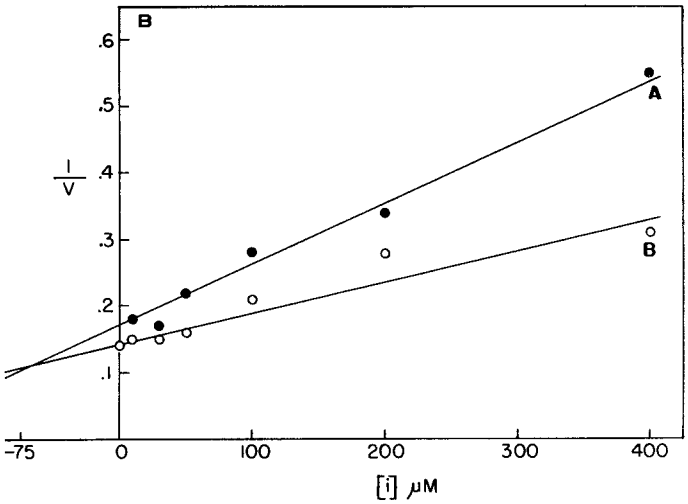


FIG. 12. *A*: K_i determination for γ-glutamylhydrazide inhibition of glutamine binding to purified glutamine binding protein. Binding assays performed as outlined in "Methods." *A*, 5 μM glutamine; *B*, 10 μM glutamine. *B*: K_i determination for γ-glutamylhydrazide inhibition of glutamine transport. Transport assays performed as outlined in "Methods." *A*, 5 μM glutamine; *B*, 10 μM glutamine.

In the presence of azaserine the internal concentration of glutamine at steady state was approximately 4×10^{-3} M, based on an estimate of cell water of 0.73 μl per mg, wet weight (31). This represents a 400-fold concentration of glutamine over the external medium.

Under a variety of conditions, parallel changes in the initial rate of transport and the level of binding protein were observed. Thus, osmotic shock caused a 90% decrease in initial rate of transport correlated with the release of binding protein. In the mutant, GLNP 1, which can use glutamine as sole carbon source, the initial rate of transport was increased 3-fold, together with a comparable 3-fold increase in level of binding protein. Starting from strain GLNP 1, a second mutant, GH 20, was isolated on the basis of resistance to γ-glutamylhydrazide. Strain GH 20 had only 3% of the initial rate of uptake and about 5% of the

TABLE VI

Effect of energy inhibitors on glutamine transport in E. coli strain GLNP 1

Cells were grown in minimal medium ("Methods") supplemented with 1% sodium succinate. They were harvested at a density of 8×10^8 cells per ml, washed three times with minimal medium, and suspended in 20 volumes of the same medium. After being incubated for 2 hours at 37° without a carbon source, the cells were centrifuged and resuspended in an equal volume of 0.12 M Tris-HCl, pH 8.0 (Tris cells). A fraction of the suspension was treated with 8 mM EDTA, incubated at 37° for 2 min, and diluted in 10 volumes of minimal medium (Tris-EDTA cells) (32).

Carbon source	Inhibitor	Concentration	Uptake remaining
			%
Tris			
Glucose			100
Succinate			100
Succinate	Dinitrophenol	2 mM	1
Succinate	FCCP[a]	10 μM	1
Succinate	KCN[b]	10 mM	1
Tris-EDTA			
Glucose			100
Succinate			100
Glucose	Dinitrophenol	2 mM	2
Glucose	DCCD[c]	50 μM	73
Glucose	Azide	10 mM	50
Glucose	FCCP	10 μM	20
Glucose	KCN	10 mM	41
Succinate	Dinitrophenol	2 mM	2
Succinate	DCCD	50 μM	41
Succinate	Azide	10 mM	41
Succinate	FCCP	10 μM	3
Succinate	KCN	10 mM	10

[a] Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.
[b] Potassium cyanide.
[c] Dicyclohexyl carbodiimide.

binding protein of strain GLNP 1 (Table V). Actually, the glutamine uptake seen in GH 20 is probably due to another system for it is inhibited completely by a 10-fold excess of nonradioactive glutamate. This component is not detected in wild type cells because of the low initial rate (1 nmole per min per mg of protein) and unfavorable K_m (6 to 8 μM), compared with the specific glutamine transport system.

When strain 7 was grown on a rich medium consisting of 3% yeast extract and 4% Tryptone, the initial rate of glutamine uptake fell to ⅓ of that observed with minimal salts medium. The level of binding protein fell by the same fraction.

Energy Requirements for Glutamine Uptake—The uptake of labeled glutamine was usually stimulated 2-fold in the presence of 1×10^{-2} M glucose, glycerol, or succinate. Cells grown on synthetic medium supplemented with glycerol showed a 10-fold stimulation by an exogenous energy source after the following treatment: incubation at 37° in synthetic medium for 2 hours, without a carbon source, followed by storage for 2 days at 3°, followed in turn by a second 2-hour incubation at 37°. In the presence of glucose, the active transport of glutamine was equal to that of fresh cells.

Experiments on the effect of inhibitors and uncouplers of oxidative phosphorylation are shown in Table VI. The uptake of [^{14}C]glutamine was inhibited by dinitrophenol, cyanide, azide, dicyclohexyl carbodiimide, and carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone.

DISCUSSION

The transport of L-glutamine is one of the most active of the bacterial amino acid uptake systems; in GLNP 1 it exceeds 30 nmoles transported per min per mg of bacterial protein. Further, the transport is highly specific; no other naturally occurring amino acid competes for uptake. In this *E. coli* mutant we find a shock-releasable binding protein present in unusual abundance, and the binding protein is specific for glutamine. γ-Glutamylhydrazide and γ-glutamylhydroxamate competitively inhibit both binding and transport with similar K_I values. Osmotic shock causes the loss of glutamine uptake but does not affect the transport of glycine, for which no binding protein is released. These facts suggest that the binding protein has a role in the active transport of glutamine. Other data also support this idea. Membrane vesicles (33, 34) do not transport glutamine, arginine, and other amino acids for which there is effective release of binding protein by osmotic shock, but they do show good uptake for glycine, proline, and the lysine-specific system (35) for which a binding protein is not ordinarily released.

Some current hypotheses picture binding proteins as carriers which undergo reversible conformational changes. Our fluorescence studies indicate that tryptophan undergoes an environmental change on binding. Tryptophan itself is not involved in the binding reaction, as oxidation with *N*-bromosuccinimide does not affect the binding. The tryptophans appear to enter a more hydrophobic environment, and this may be due to the residue becoming buried during the binding reaction. This possibility is under further investigation.

The stopped flow kinetic studies allowed us to determine the kinetic parameters of the binding reaction. The value for k_{on} of 9.8×10^7 M^{-1} sec^{-1} is about an order of magnitude slower than a diffusion-controlled reaction. Theories for a carrier model in transport suggest a rapid on reaction and these studies give the first determination of this kinetic parameter. In addition it is now possible to study environmental changes such as pH in kinetic experiments rather than equilibrium experiments. Stopped flow techniques will also allow study of the mechanism of binding. In addition, chemical modification studies are presently being used to study the active site amino acids.

The stopped flow experiments yielded a K_D of 0.16 μM, equilibrium dialysis gives a K_D of 0.3 μM, and titration of the tryptophan fluorescence at 340 nm gives a K_D of 0.3 μM. It is interesting to note that these three techniques give values which agree well with each other.

Preliminary experiments show that various inhibitors and uncouplers of oxidative phosphorylation depress the initial rate of glutamine uptake, and stimulation is observed in starved cells by an exogenous source of energy. This work is being continued for several reasons. We wish to reduce even more the stores of endogenous energy so that transport becomes rigidly dependent on whatever compound is added to the medium. In addition, we are concerned about a permeability barrier for some of the inhibitors, and how best to overcome it. Thus, it was observed that *E. coli* became much more sensitive to dicyclohexyl carbodiimide after treatment with EDTA (32) to increase permeability (Table VI). We worry about possible nonspecific toxic effects of these various compounds, especially in the presence of EDTA. Other means of increasing cell permeability are being investigated.

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