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Regulation of Glutamine Transport in *Escherichia coli*

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The formation of the high-affinity (K_m = 0.2•μM) L-glutamine transport system of *Escherichia coli* strain 7 (Lin) appears to be subject to the same major control as the glutamine synthetase (EC 6.3.1.2) of this gram-negative organism. Culture of cells under nitrogen-limited conditions provides maximum derepression of both the glutamine synthetase and the glutamine transport system. Nutritional conditions providing a rich supply of ammonium salts or available sources of nitrogen, i.e., conditions which repress the formation of glutamine synthetase, provide three- and 20-fold repression, respectively, of the glutamine transport system. Culture of cells with glutamine supplements of 2 mM does not increase the repression of high-affinity glutamine transport system beyond the level observed in the absence of glutamine. A second kinetically distinct low-affinity component of glutamine uptake is observed in cells cultured with a glutamine-depleted nutrient broth. This second component is associated with the appearance of glutaminase A (EC 3.5.1.2) and asparaginase I (EC 3.5.1.1), a periplasmic enzyme. Parallel changes were observed in the levels of the high-affinity glutamine transport system and the glutamine synthetase when cells were cultured with the carbon sources: glucose, glycerol, or succinate.

Because of the central role of glutamine synthetase activity in the nitrogen metabolism of *Escherichia coli*, considerable attention has been focused on the regulation of this enzyme as well as other enzymes directly concerned with the interconversion of glutamate and glutamine in *E. coli* (see reference 9 for review). The synthesis of glutamine is subject to: (i) specific control by induction and repression; (ii) generalized positive and negative control by cyclic adenosine 5'-monophosphate (cAMP); and (iii) constitutive control, where enzyme synthesis is independent of nutritional conditions. Generalized regulation (18) causes only a two- to threefold change in the the enzyme levels concerned with glutamine metabolism. In contrast, specific regulation may alter the level of enzyme synthesis up to 20-fold and obscure the influence of general regulation (18). In addition, at least three other forms of regulation are also operative in the enzymatic formation of glutamate and glutamine in *E. coli*: (i) allosteric feedback inhibition by specific end products or energy metabolites; (ii) covalent modification of glutamine synthetase by adenylation via a cascade system of several other enzymes and regulatory proteins which in turn are modified by precursors, products, and energy metabolites; and (iii) divalent cation modulation of enzyme activity directly and through formation with adenosine 5'-triphosphate (9).

In light of all the attention paid to the control and physiological requirements for glutamine synthesis, it is surprising that little is known about the systems responsible for the regulation of glutamine uptake and the maintenance of the endogenous pool of this amino acid. A transport system with a K_m for glutamine of 1.5×10^{-7} M has been characterized in *E. coli* strain 7 by Weiner et al. (23, 24). The high-affinity glutamine transport system was found to be specific for glutamine and repressed in cells cultured in a nutrient broth medium. When the activity of the high-affinity transport system was repressed or reduced via mutation, a second component of glutamine uptake could be demonstrated kinetically. Since very little glutamine survives autoclaving during the preparation of complex nutrient media (13), it might be expected that unlike amino acid transport systems which are repressed by their respective substrates (10, 16, 19), the high-affinity transport system is repressed by metabolites other than glutamine. This study examines the nutritional regulation of the high-affinity glutamine transport system of *E. coli* K-12 in terms of the regulation shown to occur for enzymes involved in glutamine metabolism.

MATERIALS AND METHODS

Materials. Uniformly labeled L-[^{14}C]glutamine

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was obtained from New England Nuclear. The isotope specific activity was routinely diluted to 70 mCi/mmol with unlabeled glutamine. Reduced nicotinamide adenine dinucleotide phosphate, AMP, and γ-glutamyl hydroxamate were from Sigma Chemical Co., and γ-L-glutamyl hydrazide was obtained from Nutritional Biochemicals Corp. Membrane filters (16-mm diameter) were punched from sheets (35 by 55 cm) of Selectron B6 0.45-μm nitrocellulose membrane (Schleicher and Schuell Co.).

Growth of cells. The bacteria used were obtained from L. A. Heppel, Cornell University. Strain 7 is a derivative of *E. coli* K-10 (12). Medium AS (27) contains 1% potassium succinate as the carbon source and 50 mM ammonium chloride as the nitrogen source. Where indicated, 1% glycerol or 4% glucose replaced the succinate and 17 mM L-glutamate replaced ammonium chloride. Other media were supplemented with 2 mM L-glutamine (sterilized by Millipore filtration), 8 g of Difco nutrient broth per liter, or 20% refluxed nutrient broth as noted. To insure the total conversion of glutamine to glutamate, refluxed nutrient broth was prepared as follows. Difco nutrient broth (8 g) was dissolved in 50 ml of water, and 50 ml of 2 N HCl was added. The solution was refluxed for 90 min. The cooled solution was adjusted to pH 7 with a saturated sodium hydroxide solution, and the volume was adjusted to 200 ml with distilled water. The medium was filtered through Whatman no. 1 filter paper. Solid media were prepared with 1.5% agar (Difco). Forty-milliliter cultures were grown in 250-ml Erlenmeyer flasks maintained at 37 C in a rotating shaker-bath oscillating at 250 strokes per min. Cultures were harvested in mid- to late exponential phase of growth (absorbancy at 700 nm of 0.6 to 0.8) by centrifugation at room temperature.

The cell pellets were prepared for study by three wash cycles of resuspension and centrifugation with fresh buffer A which contained 50 mM potassium phosphate (pH 7) and 0.5 mM $MgSO_4$. After the last wash, the cells were resuspended to approximately 5 mg of cellular protein per ml in buffer B (buffer A containing 300 μg of chloramphenicol per ml). The cell suspension was maintained at room temperature in a flask allowing a surface area (square centimeters) to volume (cubic centimeters) ratio of at least 1:1. Cells prepared by this method maintained constant transport and enzymatic activity for as long as 6 h.

Transport assays. Transport assays were performed at room temperature as follows: 20 μl of buffer B containing an appropriate concentration of cells was incubated for at least 10 min with 20 μl of buffer A containing 3% glycerol. The assay was initiated with 20 μl of buffer A containing isotope at three times final concentration. The assay was terminated by collecting cells from 25 μl of the assay mixture by filtration onto a nitrocellulose filter. The filters were washed immediately with 2 ml of buffer A, blotted with filter paper, and dried on a hot plate, and radioactivity was determined by liquid scintillation counting in 2 ml of toluene scintillation solution (24). Cell concentrations were adjusted before assay, such that uptake was linear for 30 s and less than 10% of the substrate was consumed from the medium during the course of assays using initial glutamine concentrations of 0.2 and 2 μM with exceptions as noted. The concentration dependence of glutamine uptake was measured with initial concentrations of glutamine ranging from 0.05 to 20 μM. Apparent K_m and V_{max} values were graphically determined following Hanes linear transformations of data (5; see Fig. 1) as suggested by Dowd and Riggs (6).

To determine if *E. coli* glutamine transport is subject to nutritional control, cells were cultured in minimal media containing either succinate, glycerol, or glucose as the carbon source, different nitrogen sources, and in the presence and absence of glutamine as indicated.

Enzyme assays. Cells (1 ml of cooled cell suspensions containing 5 mg of protein per ml of buffer B) were prepared for various enzyme assays by either sonic treatment with a Biosonik III equipped with a microtip by 4- to 15-s bursts at a power setting of 30 with intermittant 30-s cooling in an ice bath; or toluenization (0.5-ml cell suspension containing 5 mg of protein per ml of buffer B) by incubation at 37 C for 15 min with 50 μl of toluene and 5 μl of 1 M manganese chloride. After either treatment, cell suspensions were maintained in an ice bath until used. Glutamine synthetase was measured by transferase assay as described by Woolfolk et al. (28), except that toluenized, manganese-treated cell suspensions were used. The inhibition by AMP of the transferase activity provides an estimation of nonadenylated or active enzyme (9). Glutaminase A and B activities were measured as described by Meister (15) with appropriate pH considerations (17). Asparaginase EC I activity was determined with sonically treated cells as described by Campbell et al. (4). Glutamic dehydrogenase (TPN-linked) activity was measured with sonically treated cells as described by Fahien and Cohen (10). Protein values were determined by a micromodification of the method of Lowry et al. (14) with bovine serum albumin (Sigma Chemical Co.) as the standard.

RESULTS

Effect of nitrogen source on glutamine transport and glutamine synthetase levels. Conditions which result in the greatest derepression or glutamine synthetase activity also provide the highest activity measured for the high-affinity glutamine transport system (Table 1). Cells cultured in medium containing glycerol and a poor source of nitrogen, such as glutamate, have a sevenfold-higher specific activity for glutamine synthetase than cells cultured in similar media containing a nonlimiting concentration of ammonium ion. Similarly, the high-affinity glutamine transport activity of cells cultured with glutamate as the nitrogen source was threefold higher than the the activity observed with cells cultured with ammonia as the nitrogen source. When cells were cultured in a medium rich in sources of nitrogen including ammonia but depleted of glutamine (glycerol-refluxed nutrient broth), the specific activity of

TABLE 1. *Repression of glutamine transport activity with rich sources of ammonium*

Nitrogen source	Transport V_{max}[a]	Glutamine synthetase[b]		Glutamic dehydrogenase[b]	Glutaminase		Asparaginase[b]
		-AMP	50 mM AMP		A	B	
Potassium glutamate (17 mM)	54.5	0.72	0.19 (27%)[c]	0.55	0.16	0.39	0.008
Ammonium chloride (50 mM)	17.1	0.1	0.04 (40%)	1.26	0.025	0.49	0.032
Refluxed nutrient broth (0.8%)	2.8	0.085	0.037 (44%)	0.3	0.18	0.38	0.054

[a] V_{max} calculated for the 0.15 μM K_m transport system, nanomoles per minute per milligram of protein.
[b] Micromoles per minute per milligram of protein.
[c] Percentage of activity remaining in the presence of AMP.

glutamine synthetase was repressed to a level corresponding to approximately 10% of the activity observed with cells cultured in the glycerol-glutamate media. The high-affinity glutamine transport system was also repressed to 5% the level found with glycerol-glutamate-cultured cells. There appears to be little relationship associated with changes in nitrogen source and the activities of the other enzymes measured with the possible exception of the periplasmic asparaginase. The specific activity of the asparaginase appears to be influenced by the nitrogen source in a reciprocal manner to the high-affinity glutamine transport system.

A second component of glutamine transport was observable in cells with reduced uptake of glutamine via the high-affinity glutamine transport system. This lower-affinity component has an apparent K_m greater than 2 μM and is readily apparent in the linear transformation of the glutamine saturation data from glycerol-refluxed nutrient broth-grown cells (Fig. 1). A similar component of glutamine uptake was observed by Wiener and Heppel in mutants deficient in the high-affinity glutamine transport system (24). We have not been able to determine whether this component is also present in the cells with increased levels of the high-affinity glutamine transport due to the masking of this low-affinity system by the rather high velocity of the high-affinity glutamine transport system.

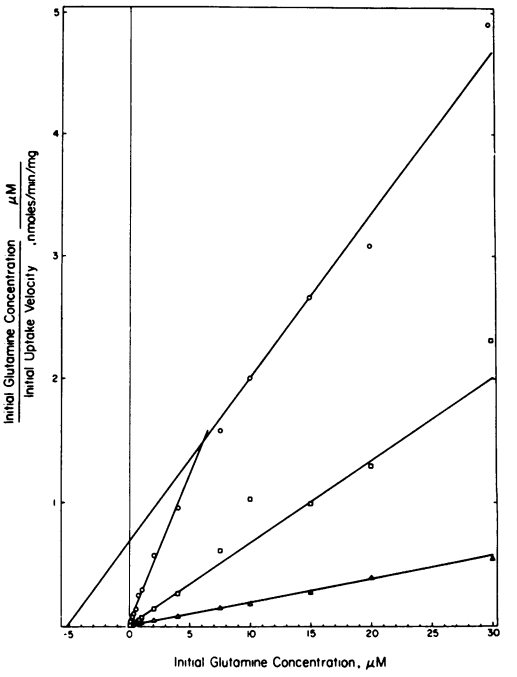

FIG. 1. *Reciprocal plot of the glutamine transport data for cells cultured in the following media: Δ, glycerol-glutamate; □, succinate-ammonia; O, refluxed nutrient broth. The details of the growth conditions and transport assays are described in the text.*

Effect of carbon source on glutamine transport and glutamine synthetase levels. To determine the effect of carbon source on the levels of the high-affinity glutamine transport system and the glutamine synthetase, cells were grown on the carbon sources listed in Table 2. The level of glutamine transport activity is observed to vary in nearly the same manner as the levels of glutamine synthetase and glutamic dehydrogenase when cells are cultured in media containing ammonium salts as the nitrogen source and different carbon sources. Cells grown with glycerol as carbon source had the highest levels of these two activities.

Effect of glutamine on glutamine transport and glutamine synthetase levels. To determine the effect of exogenously added glutamine on the levels of the high-affinity glutamine permease and glutamine synthetase, cells were grown in succinate-ammonia minimal medium with and without glutamine (Table 3). Glutamine did not repress the high-affinity glutamine transport activity of strain 7 cells in cultures grown under these conditions. An increase in the levels of glutamine synthetase and gluta-

TABLE 2. *Effect of carbon source on glutamine transport activity*

Carbon source	Transport V_{max}[a]	Glutamine synthetase[b]		Glutamic dehydrogenase[b]	Glutaminase[b]		Asparaginase[b]
		-AMP	50 mM AMP		A	B	
Glucose (4%)	10.7	0.072	0.028 (38%)[c]	1.1	0.014	0.34	0.012
Glycerol (1%)	17.1	0.1	0.040 (40%)	1.26	0.025	0.49	0.032
Potassium succinate (0.4%)	10.0	0.068	0.025 (36%)	0.53	0.15	0.33	0.022

[a] V_{max} calculated for the 0.15 μM K_m transport system, nanomoles per minute per milligram of protein.
[b] Micromoles per minute per milligram of proteins.
[c] Percentage of activity remaining in the presence of AMP.

TABLE 3. *Effects of glutamine supplements on glutamine transport activity*

Medium	Glutamine supplement (mM)	Transport V_{max}[a]	Glutamine synthetase[b]		Glutamic Dehydrogenase[b]	Glutaminase[b]		Asparaginase[b]
			-AMP	50 mM AMP		A	B	
Succinate/ammonia minimal medium	0	10.0	0.068	0.024 (36%)[c]	0.80	0.031	0.37	0.037
	2	9.4	0.130	0.059 (45%)[c]	0.53	0.15	0.31	0.022

[a] V_{max} calculated for the 0.15 μM K_m transport system, nanomoles per minute per milligram of protein.
[b] Micromoles per minute per milligram of protein.
[c] Percentage of activity remaining in the presence of AMP.

minase A were observed, however, and a greater percentage of the glutamine synthetase appeared to be in the inactive form as judged from the AMP inhibition.

DISCUSSION

The study of the regulation of amino acid permease levels is complicated by a number of factors. One of the least appreciated difficulties arises from the multiplicity of transport systems for a given substrate. For example, histidine appears to enter the cell through at least five different transport systems (1); the branched-chain amino acids appear to utilize at least three closely related binding protein-associated systems (8; R. C. Willis, N. H. Gerber, C. E. Furlong, Abstr. 9th Int. Cong. Biochem., p. 278, 1973) and aspartic acid enters the cell through more than one system (11), one of which appears to be a binding protein-dependent system (26). Additional complications arise from specific transport systems which are either repressed (10, 13, 16, 19) or induced (2, 3) by the presence of the substrate in the medium. Further, catabolite repression also appears to play a role in regulating some of the amino acid permeases (3).

The purpose of this study was to investigate the regulation of the high-affinity glutamine transport system. At low concentrations of substrate, the transport of glutamine does not appear to be complicated by the existence of more than one transport system (24).

Our results indicate that the formation of the high-affinity glutamine transport system of *E. coli* strain 7 is neither induced nor repressed by exogenous glutamine. The activity is to some extent dependent on the carbon source used in culture. It has been demonstrated by Prusiner et al. (17) that cyclic AMP exerts a parallel and positive control over the formation of glutamine synthetase and glutamic dehydrogenase and a negative control over the formation of glutaminase A. The data shown in Table 2 are consistent with the interpretation that the level of cyclic AMP in the cell may, in part, control the formation of the high-affinity glutamine transport system.

The major control of the high-affinity glutamine permease appears to be related to the availability of nitrogen. Further, the pattern of regulation observed is qualitatively similar to that found for glutamine synthetase in this and other studies (17, 18, 20, 21, 28). Derepression of the transport system and the synthetase occurs during the culture of cells in media limited in available nitrogen. Ammonium ion effects a partial but significant repression of the transport system while causing a near total repression of the synthetase. Maximum repression of both activities occurs with culture in media rich in sources of nitrogen including ammonium ion but lacking glutamine. Under the latter condition, the repressed levels of the synthetase and transport activity remain physiologically significant since the cells are required to synthe-

size and maintain a pool of glutamine for protein synthesis.

The range of repression observed for the transport system appears to be significant since the extremes of activity are equivalent to levels of glutamine transport in *E. coli* mutants with elevated or diminished levels of glutamine transport reported by Wiener and Heppel (24). Based on our observations, mutants such as those described could result from a mutation in the regulatory mechanism(s), as well as in the structural gene(s) of the transport system.

Substrate-dependent repression of an active transport system can be understood if, as has been suggested (29), transport systems assist in maintaining the level of the endogenously synthesized pool of substrate (29) or spare the requirement for de novo synthesis of the substrate by scavenging from the environment. An increase in the external supply of the substrate decreases the need for active pool maintenance and scavenging. This, in turn, effects a repression of both the transport and biosynthetic systems for the substrate.

The apparent repression of the glutamine transport system by nitrogen is perhaps not as obvious. In addition to being a necessary component for protein synthesis, glutamine occupies a central position as a biosynthetic intermediate interchangeable with ammonium ion in the synthesis of many compounds (9). The requirement for glutamine is apparently determined by the availability of ammonium or nitrogen sources convertible to ammonium. The synthesis of glutamine from glutamate serves as an effective means of sequestering ammonia to prevent the loss to the medium.

When the availability of nitrogen from the medium is low, the cells may have both a high level of glutamine synthetase and a high level of the high-affinity glutamine permease to insure that ammonia nitrogen and consequently glutamine synthesis from this nitrogen are maintained. Under conditions where ammonia is readily available from the medium, it may not be necessary to either scavenge or maintain high levels of glutamine. Further, a high level of the glutamine permease under culture conditions which allow maximum derepression of the synthetase may also assure that a high-glutamine pool be maintained for a sufficient time to activate the conversion of glutamine synthetase to the inactive, adenylated form should the available nitrogen suddenly increase in the cell's environment (20).

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