JOURNAL OF BACTERIOLOGY, June 1995, p. 3150–3157 Vol. 177, No. 11
0021-9193/95/$04.00+0
Copyright © 1995, American Society for Microbiology

Amino Acid Transport Systems Required for Diazotrophic Growth in the Cyanobacterium *Anabaena* sp. Strain PCC 7120

MARÍA LUZ MONTESINOS, ANTONIA HERRERO, AND ENRIQUE FLORES*

Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-Consejo Superior de Investigaciones Científicas, E-41080 Seville, Spain

Received 6 February 1995/Accepted 30 March 1995

Uptake of 16 amino acids by the filamentous, heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 was characterized with regard to kinetic parameters of transport, intracellular accumulation of the transported amino acids, and sensitivity of the transport process to energy metabolism inhibitors. Mutants resistant to certain toxic analogs of some amino acids were isolated that were impaired in amino acid transport. Results obtained in this study, together with those reported previously (A. Herrero and E. Flores, J. Biol. Chem. 265:3931–3935, 1990), suggest that there are at least five amino acid transport systems in strain PCC 7120: one high-affinity, active system for basic amino acids; one low-affinity, passive system for basic amino acids; two high-affinity, active systems with overlapping, but not identical, specificities for neutral amino acids; and one putative system for acidic amino acids. Some of the amino acid transport mutants were impaired in diazotrophic growth. These mutants were unable to develop a normal percentage of heterocysts and normal nitrogenase activity in response to nitrogen stepdown. Putative roles for the amino acid transport systems in uptake of extracellular amino acids, recapture of amino acids that have leaked from the cells, and intercellular transfer of amino acids in the filaments of *Anabaena* sp. strain PCC 7120 are discussed.

The cyanobacteria are a phototrophic group of eubacteria that carry out oxygenic photosynthesis. Most cyanobacteria can use nitrate and ammonium as nitrogen sources, and many are also able to fix atmospheric nitrogen (dinitrogen). The enzyme complex that fixes dinitrogen, nitrogenase, is highly sensitive to oxygen. Nitrogen fixation and oxygenic photosynthesis are generally separated, either temporally or spatially, in cyanobacteria (5). When grown under aerobic conditions, some filamentous cyanobacteria such as those of the genus *Anabaena* confine nitrogenase to cells specialized in nitrogen fixation, the heterocysts, where *nif* genes are expressed (36). The heterocysts are incapable of photosynthetic CO_2 fixation and are supplied with reduced carbon by the adjacent vegetative cells (32). Vegetative cells receive in turn fixed nitrogen from the heterocysts (34). Although ammonium is the immediate product of nitrogen fixation by nitrogenase, the heterocyst appears to transfer fixed nitrogen to neighboring vegetative cells in the form of amino acids (37). Heterocysts are able to produce glutamine (30), but the identity of the amino acid(s) transferred from heterocysts to vegetative cells has not been established (36). Some amino acids could also be involved in the transfer of reduced carbon from the vegetative cells to the heterocysts (14).

The mechanism of intercellular transfer of metabolites in the heterocyst-forming cyanobacteria is unknown. Diffusion through thin structures termed microplasmodesmata that have been observed between adjacent cells in filamentous cyanobacteria (see, e.g., reference 10) is commonly assumed, but experimental evidence for this is not available. The interesting working hypothesis that transport systems or permeases mediate metabolite transfer between cells in cyanobacterial filaments has been raised. It has been conjectured that prior to heterocyst development, upon nitrogen stepdown, some amino acid "pumps" might function, leading to the establishment of concentration gradients of amino acids in the filaments (11, 33).

Information on amino acid transport in heterocyst-forming cyanobacteria is scanty and disperse. In *Anabaena* sp. strain PCC 7120, there are both high-affinity (active) and low-affinity (passive) transport systems for basic amino acids; mutants lacking the high-affinity system that show a low-affinity activity of uptake of basic amino acids have been isolated (12). Strain PCC 7120 has also been shown to be able to take up Pro (26), to transport Gln and Glu probably by means of a common permease (7), and to take up Phe through a high-affinity system (38). In *Anabaena variabilis*, high- and low-affinity systems for the transport of Gln and Glu (4) as well as a system taking up Leu (29) have been reported. High-affinity transport of Gln has also been detected in *Anabaena cylindrica* (24), and a Glu/Asp transport system has been detected in a *Nostoc* sp. (28). In contrast to the basic amino acid transport systems of strain PCC 7120, which are specific for basic amino acids (12), the transport systems taking up Gln and Glu (4, 7), Leu (29), and Phe (38) might bear broad specificities, since transport of Gln, Glu, Leu, and Phe is inhibited by many other amino acids.

Within the context of diazotrophic metabolism in cyanobacteria, we considered the possibility that amino acid permeases were involved in intercellular transfer of nitrogen in heterocyst-forming strains. In this work, we attempted to gain a general view of amino acid transport in a heterocyst-forming cyanobacterium. Uptake of 16 radioactively labeled amino acids has been studied, and amino acid transport mutants have been isolated and analyzed. Additionally, the mutants have been used to address the question of a possible role of amino acid permeases in diazotrophic growth.

MATERIALS AND METHODS

Strains and growth conditions. *Anabaena* sp. strain PCC 7120 was grown axenically in BG11 (nitrate-containing) medium (23), $BG11_0$ (nitrogen-free) medium, or $BG11_0$ medium supplemented with 2.5 mM NH_4Cl and 8 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-NaOH buffer

* Corresponding author. Mailing address: Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-Consejo Superior de Investigaciones Científicas, Apartado 1113, E-41080 Seville, Spain. Phone: 34-5-4557086. Fax: 34-5-4620154. Electronic mail address: Flores@CICA.ES.

Downloaded from http://jb.asm.org/ on July 25, 2017 by INSTITUTO DE PARASITOLOGFA Y

(pH 7.5). For plates, the medium was solidified with 1%, separately autoclaved agar (Difco). Cultures were grown at 30°C in the light, with shaking (80 to 90 rpm) for liquid cultures. Strain EF116 is a mutant derived from strain PCC 7120 that bears a defective heterocyst envelope and is unable to fix dinitrogen under aerobic conditions (35).

Amino acid transport mutants CS326 and CS327 are canavanine-resistant mutants derived from PCC 7120 that had been isolated previously (12). The following mutants were isolated in this work: strain CS343, is an azaleucine-resistant mutant derived from strain PCC 7120; strain CS349, is an azaserine-resistant mutant derived from strain CS343; and strain CS389, a methionine sulfoximine-resistant mutant derived from strain CS343. Two mutants resistant to ethionine derived from strain PCC 7120, strains CS341 and CS342, were also used. The mutants were grown in BG11 medium supplemented with an amino acid analog. Amino acid analogs (from Sigma) were used for the mutant strains at the indicated concentrations, which were inhibitory for the wild-type strain: L-canavanine sulfate (50 μM) or δ-hydroxylysine (50 μM) for strains CS326 and CS327, 4-aza-DL-leucine (100 μM) for strain CS343, azaserine (50 μM) for strain CS349, L-methionine sulfoximine (50 μM) for strain CS389, and DL-ethionine (100 μM) for strains CS341 and CS342. These amino acid analogs are antagonists of the following amino acids: azaleucine, Leu; azaserine, Gln; canavanine, Arg; ethionine, Met; hydroxylysine, Lys; and methionine sulfoximine, Met and Glu. The amino acid analogs were filter sterilized. L-Canavanine sulfate was neutralized with NaOH before filtration.

To determine the abilities of amino acid transport mutants to grow with dinitrogen as the nitrogen source, cells that had been grown on BG11 medium in the presence of the amino acid analog adequate for each mutant were washed with $BG11_0$ and used to inoculate flasks with 50 ml of $BG11_0$ at 0.2 μg of chlorophyll *a* (Chl) per ml. (Chl was determined in methanolic extracts [18].) Samples of 0.2 ml were withdrawn and frozen at different times. The amount of protein of these samples was determined by a modified Lowry procedure (19), using bovine serum albumin as the standard. The amount of cells containing 1 μg of Chl contains about 25 to 30 μg of protein.

Mutant isolation. Spontaneously occurring mutants were isolated from BG11 plates supplemented with an inhibitory concentration (see above) of azaleucine, azaserine, methionine sulfoximine, or ethionine. The plates were inoculated with ca. 10 μg of Chl (about 3×10^7 cells) in soft (0.5%) top agar, and individual colonies that appeared about 1 week after inoculation were restreaked on selective medium.

Uptake assays. Unless otherwise indicated, cells grown on BG11 medium (supplemented with the appropriate amino acid analog in the case of the mutants) were harvested by low-speed centrifugation at room temperature, washed with 25 mM *N*-tris(hydroxymethyl)-methylglycine (Tricine)-NaOH buffer (pH 8.1), and resuspended in the same buffer. The assays were carried out at 30°C in the light (100 $W \cdot m^{-2}$, white light from incandescent lamps) and were started by mixing a suspension (1 ml) of cells (5 to 13 μg of Chl) with a solution (0.1 ml) in Tricine buffer of the amino acid to be tested as a substrate. The amino acids used as substrates were L-U-^{14}C-labeled amino acids (7.7 to 70 μCi/μmol), except in the case of methionine (L-[^{35}S]methionine, 84 to 187 μCi/μmol), at a concentration of 110 μM (10 μM in the assay). Total reaction volume was 1.1 ml. After incubation for 10 min, a 1-ml sample was filtered (0.45-μm-pore-size Millipore HA filters were used) and washed with 5 to 10 ml of Tricine buffer. The filters were then immersed in scintillation cocktail, and their radioactivity was measured. Retention of radioactivity by boiled cells was used as a blank.

To study linearity of uptake in nitrate-grown cells of strain PCC 7120, uptake assays were started by the addition of 0.5 ml of 170 μM radioactively labeled amino acid (22.5 to 45 μCi/μmol) to an 8-ml cell suspension (ca. 9 to 15 μg of Chl per ml), resulting in an amino acid concentration in the assays of 10 μM. Samples of 1 ml were withdrawn, filtered, and washed at different times (1, 3, 6, 10, 15, 20, and 30 min) after the start of the assay.

Uptake assays for the determination of K_s (solute concentration that gives one-half of the maximum rate of uptake) and V_{max} values in wild-type strain PCC 7120 and mutant CS343 were carried out using the following substrate concentrations: 0.5 to 20 μM for Gly, 15 to 800 μM for Asp, 50 to 2,000 μM for Glu, and 5 to 400 μM for the other amino acids tested. The assays were started by the addition of 0.25 ml of a cell suspension (9 to 12 μg of Chl) in Tricine buffer to a solution of substrate in the same buffer. Radioactivity used was 1.5 to 3.7 μCi/μmol (^{14}C), except for Gly (36.6 μCi/μmol, ^{14}C), Glu (0.6 μCi/μmol, ^{14}C), and Met (8.4 μCi/μmol, ^{35}S). Total reaction volume was 1.05 ml, and the incubation time was 1 min. K_s and V_{max} values were estimated from Lineweaver-Burk plots of the data obtained in uptake assays with different amino acid concentrations. Lineweaver-Burk plots giving straight lines with $r^2 > 0.9$ were always used. Two or three determinations, with similar results (within 20% of the mean), were carried out for each amino acid; for the sake of simplicity, the data are presented as the mean of the values obtained in those determinations.

The effects of *N,N'*-dicyclohexylcarbodiimide (DCCD) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on uptake of individual amino acids were studied in nitrate-grown cells of strain PCC 7120. Cells (ca. 13 μg of Chl per ml of Tricine buffer) were supplemented with 50 μM DCCD or 25 μM CCCP and incubated for 15 min in the light at 30°C. The assays were then started by the addition of 0.1 ml of a concentrated solution of L-U-^{14}C-labeled amino acid (110 μM, 18.18 μCi/μmol) to 1 ml of cell suspension. The amino acid concentration in the assay was 10 μM, and the incubation time was 10 min.

To determine the inhibitory effects of some amino acids on the transport of Asn, Leu, Met, and Thr in strain PCC 7120, the uptake of 10 μM these amino acids (radioactively labeled) was measured in the presence of 500 μM the (nonradioactive) amino acid tested as an inhibitor. The assays were started by the addition of cells (ca. 9 μg of Chl) to the solution of amino acids. Radioactivity used was 8 to 9 μCi/μmol (^{14}C) except in the case of Met (80 μCi/μmol, ^{35}S). Total reaction volume was 1.15 ml, and the incubation time was 10 min. Two independent determinations, with similar results (within 15% of the mean), were carried out for Asn and Leu transport, and the data are presented as the mean of the values obtained in those determinations. Only one determination was carried out for Met and Thr transport.

Intracellular accumulation of labeled amino acids. After uptake assays had been conducted for 11 min, washed filters containing the cells used in the assays were immediately immersed in 2 ml of boiling water and incubated at 100°C for 5 min. Each filter was then withdrawn, and the resulting suspension was centrifuged. Samples from the supernatant solutions were lyophilized and dissolved in a small volume of water. Samples of the resulting solutions were applied to 0.1-mm cellulose thin-layer chromatography (TLC) plates (20 by 20 cm; Merck). Two-dimensional separation of amino acids was effected by using the following solvents: first dimension, *n*-butanol–acetone–ammonium hydroxide–water (20:20:10:4 by volume) and second dimension, isopropanol-formic acid-water (20:1:5 by volume). X-ray films were then exposed to the TLC plates and developed after 1 week. Zones of the TLC plates responsible for the autoradiography spots were scraped and combined with a scintillation cocktail, and their radioactivity was measured. Identification of the spots was made by cochromatography, supplementing the samples with stable amino acids as markers, and visualizing the amino acids after chromatography with a solution of ninhydrin in acetone in the presence of cadmium acetate (3). To calculate the intracellular concentration of labeled amino acids, an intracellular volume of 125 μl/mg of Chl (5 μl/mg of protein) was assumed (13, 22).

Amino acids in the culture medium and intracellular pool of amino acids. Wild-type strain PCC 7120, strain EF116, and amino acid analog-resistant mutants were grown in liquid BG11 medium (with the corresponding analog added in the case of the mutant strains) for 1 week. Cells were then harvested, washed, and resuspended in BG11 or $BG11_0$ medium, without analogs, at 1 μg of Chl per ml. After 48 h of incubation under culture conditions, samples were withdrawn and filtered. A portion of the filtered solution (culture medium without cells) was frozen, lyophilized, and dissolved in a small volume of water. Amino acids present in the concentrated samples were then separated by high-performance liquid chromatography (HPLC).

Cells grown on BG11 medium were used to analyze the intracellular pool of amino acids. Cells (50 μg of Chl) were harvested, washed with Tricine buffer, and resuspended in a final volume of 2 ml of the same buffer. The cell suspensions were then boiled and centrifuged, and amino acids in the supernatant solutions were resolved by HPLC.

The method used for reverse-phase HPLC of amino acids involves derivatization of amino acids with orthophthalaldehyde and β-mercaptoethanol and then detection by fluorescence emission at 425 nm or absorbance at 340 nm. A gradient of two solvent mixtures was used (20). Solvent A contained 400 mM sodium phosphate (pH 7.2), tetrahydrofuran, and water (5:1.5:93.5 by volume); solvent B contained 400 mM sodium phosphate (pH 7.2), acetonitrile, and water (2.5:55:42.5 by volume). The gradient program used was as follows: time zero, 0% solvent B; 0.8 min, 0% solvent B; 4.2 min, 20% solvent B; 6.6 min, 20% solvent B; 8.5 min, 30% solvent B; 10.6 min, 30% solvent B; 13.3 min, 38% solvent B; 14.8 min, 40% solvent B; 15.4 min, 42% solvent B; 15.8 min, 43% solvent B; 20 min, 60% solvent B; 23 min, 0% solvent B. The Waters chromatograph used was equipped with a LiChrospher 100 RP-18 (5-μm) column (Merck). An amino acid standard solution (from Sigma) was used for calibrating the apparatus. Identification of each peak was made by cochromatography. Pro, Lys, and Cys could not be detected by this method.

In an alternative experimental approach to test the release of amino acids to the outer medium, cells of wild-type strain PCC 7120 and mutant CS326 were grown in liquid BG11 medium (supplemented with hydroxylysine in the case of CS326), harvested, and washed with $BG11_0$ medium. Tubes with 2.5 ml of BG11 were inoculated at 2 μg of Chl per ml, and 10 μCi of $NaH^{14}CO_3$ (55.5 mCi/mmol) was added. The cell suspensions were incubated under culture conditions for 74 h and filtered, and 2-ml samples of the filtrates were frozen, lyophilized, and dissolved in 100 μl of water. Amino acids present in the samples were separated and identified by two-dimensional TLC as described above.

Nitrogenase activity and frequency of heterocysts. Wild-type strain PCC 7120 and mutant strains were grown for 1 week in liquid BG11 medium either with or without added analogs. When analogs were not added, transport of some amino acids was measured in order to discard any cultures that could have accumulated revertants. Cells were then harvested, washed with $BG11_0$ medium, and resuspended in the same medium at about 1 μg of Chl per ml. Nitrogenase activity and frequency of heterocysts were determined after 48 h of incubation under culture conditions. To determine nitrogenase activity, cells (2.5 to 22 μg of Chl) from 10 ml of culture were harvested, resuspended in 2 ml of fresh $BG11_0$ medium, and incubated under an atmosphere of 13.3% acetylene in air. The amount of ethylene produced after 30 to 60 min of incubation with shaking in the light at 30°C was determined by gas chromatography. The frequency of heterocysts (number of heterocysts present among a total number of 2,500 to 6,000 cells that

Downloaded from http://jb.asm.org/ on July 25, 2017 by INSTITUTO DE PARASITOLOGFA Y

TABLE 1. Characteristics of amino acid transport in *Anabaena* sp. strain PCC 7120[a]

Amino acid	K_s (μM)	V_{max} (nmol/min/mg of Chl)	Intracellular accumulation[b]	% Inhibition by[c]: DCCD	% Inhibition by[c]: CCCP
Basic					
Arg[d]	1.7	10	200	99	85
His	43	44	40	ND[e]	ND
Lys[d]	1.9	10	ND	ND	ND
Orn	5.6	16	ND	ND	ND
Neutral					
Ala	6.8	34	52	85	79
Asn	17	16	42	95	91
Gln	38	30	17	79	71
Gly	2.0	31	128	83	82
Leu	39	67	22	61	55
Met	27	11	ND	ND	ND
Phe	44	42	45	79	77
Pro	20	35	20	95	88
Ser	12	35	107	84	82
Thr	32	56	63	ND	ND
Acidic					
Asp	132	26	5 (35)	19	24
Glu	465	37	3 (1,500)	30	30

[a] Transport assays were carried out with BG11-grown cells as described in Materials and Methods. L Isomers of amino acids were used as substrates.

[b] Ratio of intracellular to extracellular concentration of labeled amino acid after 11 min of uptake of 10 μM ^{14}C-labeled amino acid (10 min and 2 μM in the case of Arg). Note that this is a minimum estimation, since the size of the intracellular unlabeled pool of amino acids present in the cells prior to amino acid transport is not considered. This fact is significant in the case of Asp and Glu. Values in parentheses refer to the concentration gradient, taking into account the intracellular concentration of unlabeled amino acid.

[c] The percentage of inhibition by 50 μM DCCD or 25 μM CCCP of the uptake of 10 μM ^{14}C-labeled amino acid (5 μM in the case of Arg) was determined in 10-min uptake assays.

[d] Data from reference 12.

[e] ND, not determined.

TABLE 2. Effects of 22 amino acids on the transport of Asn, Leu, Met, and Thr by *Anabaena* sp. strain PCC 7120[a]

Nonradioactive amino acid added	% Inhibition of transport of: Asn	Leu	Met	Thr
Basic				
Arg	35	10	2	9
His	94	60	62	87
Lys	39	16	5	33
Orn	59	26	17	48
Neutral				
Ala	84	92	86	98
Asn		42	43	70
Citrulline	78	32	28	57
Cys	97	86	89	98
Gln	96	69	68	90
Gly	79	86	80	97
Ile	66	86	61	96
Leu	68		72	97
Met	91	94		98
Phe	66	90	59	95
Pro	67	50	29	74
Ser	92	82	85	96
Thr	74	77	72	
Trp	68	68	52	87
Tyr	67	72	48	88
Val	68	88	66	96
Acidic				
Asp	8	9	1	2
Glu	26	14	1	10

[a] The transport of 10 μM [^{14}C]Asn, [^{14}C]Leu, [^{35}S]Met, and [^{14}C]Thr was measured, in 10-min assays, in the presence of 500 μM each nonradioactive amino acid indicated.

were counted) was determined by microscopic examination of the cell suspensions.

RESULTS

Amino acid transport. Nitrate-grown cells of *Anabaena* sp. strain PCC 7120 were able to take up the amino acids listed in Table 1. When the amino acids were supplied at a concentration of 10 μM, uptake was linear for at least 10 to 15 min under the experimental conditions used (see Materials and Methods). Cells that had been grown fixing dinitrogen or using ammonium as the nitrogen source took up the amino acids listed in Table 1 (Met, Orn, and Thr were not analyzed) at rates similar to those exhibited by nitrate-grown cells (not shown).

The kinetic characteristics of transport of Arg and Lys (12), Glu and Gln (7), and Phe (38) in strain PCC 7120 have been reported previously. We have now investigated the effect of the concentration of substrate on the rate of transport for all the other amino acids listed in Table 1 (and have reinvestigated Gln, Glu, and Phe transport). Transport assays were conducted with different substrate concentrations (see Materials and Methods), saturation kinetics with a single kinetic component being observed in every case tested. From the data obtained, kinetic parameters K_s and V_{max} were derived and are summarized, along with data on the high-affinity transport of Arg and Lys (12), in Table 1. The K_s for most amino acids was within 12 to 44 μM, but for three basic amino acids (Arg, Lys, and Orn), Ala, and Gly it was lower (K_s, 1.7 to 6.8 μM), and for the acidic amino acids Asp and Glu it was higher (K_s, 132 and 465 μM, respectively). V_{max} for the transport of the different amino acids was between 10 and 67 nmol/min/mg of Chl (Table 1).

Intracellular accumulation of His and of neutral and acidic amino acids was examined after 11 min of uptake. Transport of His and neutral amino acids was concentrative (Table 1). Consistent with this result, uptake of neutral amino acids was inhibited by the ATPase inhibitor DCCD and by CCCP, a protonophore (Table 1). These results show that metabolic energy is necessary for concentrative uptake of neutral amino acids in strain PCC 7120. The active and concentrative nature of Arg transport has been shown previously (12). On the other hand, only a limited inhibition of uptake of acidic amino acids by the metabolic inhibitors DCCD and CCCP was observed (Table 1). However, cells of strain PCC 7120 were able to take up Asp and Glu against a concentration gradient, since the intracellular concentrations of these amino acids in the cells used in the uptake assays were about 0.3 and 15 mM, respectively (not shown).

To examine the specificity of the putative transport system(s) that might be involved in the transport of neutral amino acids, the effects of some amino acids on the uptake of Asn, Leu, Met, and Thr were examined. The uptake of these amino acids was notably inhibited by all of the other neutral amino acids that were tested and less inhibited by basic or acidic amino acids (Table 2). Note that His, which was inhibitory, should be mostly in a neutral form at the pH used for the uptake assays (pK_a, His side chain, 6.00).

Amino acid transport mutants. A series of spontaneous mutant strains resistant to amino acid analogs was isolated. With the wild-type strain PCC 7120 used as a parental strain, mutants resistant to azaleucine were obtained at a frequency of 1.7×10^{-6} per cell, but no mutants resistant to azaserine or to

Downloaded from http://jb.asm.org/ on July 25, 2017 by INSTITUTO DE PARASITOLOGFA Y

TABLE 3. Amino acid transport activities in amino acid analog-resistant mutants of *Anabaena* sp. strain PCC 7120[a]

Amino acid	Transport activity (%) in mutant strain[b]:				
	CS326	CS327	CS343	CS349	CS389
Basic					
Arg	1	2	100	<1	100
His	3	63	96	<1	100
Lys	<1	4	99	<1	100
Orn	<1	17	55	<1	63
Neutral					
Ala	1	100	23	1	4
Asn	3	55	46	2	2
Gln	9	71	59	2	24
Gly	<1	95	4	<1	1
Leu	1	62	7	2	1
Met	2	50	33	1	1
Phe	2	76	3	2	2
Pro	<1	92	<1	<1	<1
Ser	1	100	30	<1	1
Thr	<1	67	13	1	3
Acidic					
Asp	48	9	91	23	44
Glu	63	58	68	22	22

[a] The transport of 10 μM labeled amino acid was measured in 10-min assays. Cells of the mutant strains used in the transport assays had been grown on BG11 medium supplemented with an amino acid analog (see Materials and Methods). Data for transport in the mutants are presented as percentages of the transport values exhibited by the wild-type strain PCC 7120. The values presented are based on three to nine independent determinations.

[b] Strains CS326 and CS327 are canavanine-resistant mutants of strain PCC 7120; strain CS343 is an azaleucine-resistant mutant of strain PCC 7120; strain CS349 is an azaserine-resistant mutant of strain CS343; strain CS389 is a methionine sulfoximine-resistant mutant of strain CS343.

methionine sulfoximine could be isolated (frequencies of $<2.4 \times 10^{-9}$ and $<5.1 \times 10^{-9}$, respectively). However, when an azaleucine-resistant mutant was used as a parental strain, secondary mutants resistant also to azaserine and methionine sulfoximine were readily obtained with frequencies of 1.4×10^{-6} and 8.6×10^{-7} per cell, respectively. The uptake activities of 16 amino acids were examined in one azaleucine-resistant mutant of strain PCC 7120 (strain CS343), one azaserine-resistant derivative of CS343 (strain CS349), and one methionine sulfoximine-resistant derivative of CS343 (strain CS389); the results obtained are summarized in Table 3. We have also examined the amino acid uptake activities of two canavanine-resistant mutants of strain PCC 7120, strains CS326 and CS327, whose Arg and Lys transport activities had been studied before (12).

Strain CS326 was found to be strongly affected not only in the uptake of basic amino acids but also in the uptake of neutral and, to a lesser extent, acidic amino acids. Strain CS327 was strongly affected in the uptake of basic amino acids and Asp but less in that of neutral amino acids and Glu. Strain CS343 was impaired mainly in the uptake of neutral amino acids as well as of Glu and Orn, but the uptake of different neutral amino acids was affected to different degrees. In particular, strain CS343 was strongly affected in the uptake of Pro, Phe, Gly, Leu, Thr, and Ala. Strain CS349, a derivative of CS343, was strongly affected in the uptake of all of the tested amino acids and exhibited a phenotype close to that of strain CS326; however, rates of uptake of acidic amino acids were lower in strain CS349 than in strain CS326. Finally, strain CS389, also a derivative of CS343, was strongly affected in the uptake of neutral amino acids (though some uptake of Gln was still observed) and was clearly affected in the uptake of acidic amino acids, whereas it showed wild-type levels of uptake of basic amino acids (except for Orn). Thus, the mutations analyzed inactivated the uptake of sets of, rather than of single, amino acids.

An impairment in uptake can be the result of a mutation inactivating a transport system or of transinhibition of the transport system by intracellularly accumulated amino acid(s) in mutants with a deregulated biosynthesis of some amino acid(s). We examined the intracellular levels of amino acids in the wild-type strain PCC 7120 and in the mutants CS326, CS327, CS343, CS349, and CS389 (see Materials and Methods). The HPLC chromatograms of intracellular amino acids from BG11-grown cells were dominated by Glu, and no significant differences were observed between the wild-type and mutant strains (data not shown). This result is not consistent with the hypothesis of transinhibition and suggests that the impaired uptake of amino acids that we observed was the result of direct inactivation of some transport system(s). Additionally, two mutants (strains CS341 and CS342) resistant to ethionine, which were found to accumulate intracellularly some amino acids (Ser, Gly, Thr, Ile, and Met) at levels 5 to 100 times higher than those found in the wild-type strain, exhibited wild-type levels of amino acid transport activity (not shown).

The values of K_s for transport of some neutral amino acids were determined in mutant strain CS343 with the following results: K_s (Ala), 22 μM; K_s (Asn), 9 μM; K_s (Gln), 35 μM; and K_s (Ser), 12 μM. These K_s values are similar to those found for the same amino acids in the wild-type strain (Table 1).

Release of some amino acids. Some nitrogen-fixing cyanobacteria have been reported to excrete some amino acids into the culture medium (8, 9, 27, 31). We have tested whether wild-type strain PCC 7120 and the amino acid transport mutants accumulated amino acids in the extracellular medium. BG11-grown cells were washed and incubated in BG11 or $BG11_0$ medium for 48 h. Extracellular accumulation of some amino acids, above the levels found with the wild type, was observed to take place with some mutants. Figure 1 shows HPLC chromatograms of samples from BG11 culture media in which strains PCC 7120, CS326, and CS343 had been incubated. Amino acids released by the mutants included Ala, Val, Ile, Phe, and Leu. A summary of quantitative data obtained with the whole set of mutants is presented in Table 4. The mutants that released amino acids at levels above those observed with the wild-type strain were CS326, CS343, CS349, and CS389, whereas mutant CS327 behaved similarly to the wild-type strain. Similar results were obtained after incubation in BG11 or $BG11_0$ medium. Strain EF116, a mutant that is unable to fix dinitrogen under aerobic conditions (35), was used in this experiment as a control to ensure that amino acid accumulation in the culture medium was not a consequence of the nitrogen stress that resulted from the inability of some of the mutants to grow on dinitrogen (see below).

The results obtained by HPLC analysis of the culture media were corroborated by using an alternative experimental approach. Cells of strains PCC 7120 and CS326 were fed with [^{14}C]bicarbonate and incubated in BG11 medium for 74 h. The cell suspensions were then filtered, the filtrates were concentrated, and samples of the concentrated filtrates were subjected to two-dimensional TLC and autoradiographed (see Materials and Methods for details). Whereas no spot was produced by samples from cell suspensions of the wild-type strain, spots that cochromatographed with Ala, Phe, Ile (plus Leu), and Val were produced by samples that originated from a cell suspension of mutant CS326, the most radioactive spot being that corresponding to Ala.

Impairment in diazotrophic growth. All of the amino acid

Downloaded from http://jb.asm.org/ on July 25, 2017 by INSTITUTO DE PARASITOLOGFA Y

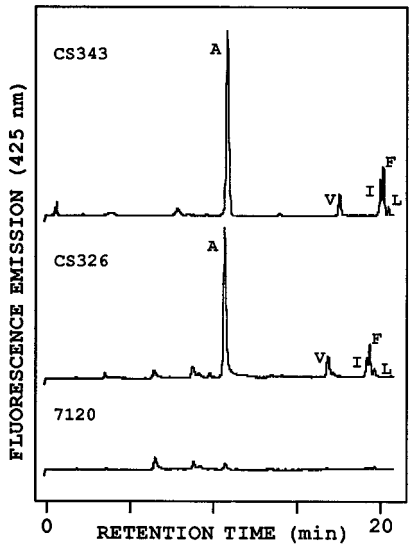

FIG. 1. HPLC chromatograms of samples from the extracellular medium of cultures of *Anabaena* sp. strain PCC 7120 and mutant strains CS326 and CS343. Cells grown on BG11 medium (supplemented with amino acid analogs in the case of the mutants) were washed, resuspended in BG11 medium, and incubated under culture conditions for 48 h. Samples of the cell suspensions were filtered, and the filtrates were concentrated and subjected to HPLC analysis as described in Materials and Methods. The amino acids detected were Ala (A), Val (V), Ile (I), Phe (F), and Leu (L).

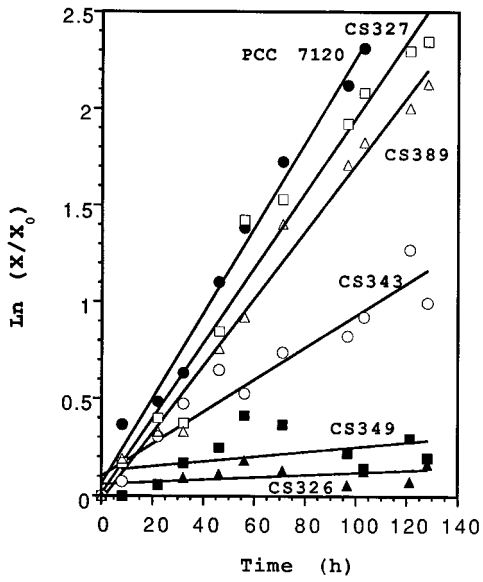

FIG. 2. Growth curves of *Anabaena* sp. strain PCC 7120 and some amino acid analog-resistant mutants on $BG11_0$ (nitrogen-free) medium. Cells of the wild-type strain PCC 7120 and the indicated mutant strains that had been grown on medium BG11 (supplemented with amino acid analogs in the case of the mutants) were washed, resuspended in $BG11_0$ medium, and incubated under culture conditions. Shown are data for wild-type strain PCC 7120 (●) and mutant strains CS326 (▲), CS327 (□), CS343 (○), CS349 (■), and CS389 (△). X, micrograms of protein per milliliter at $t = x$ h; X_0, micrograms of protein per milliliter at $t = 0$.

transport mutants described in this work grew on BG11 medium at rates similar to that of the wild-type strain PCC 7120. However, when cells of mutant strains CS326 and CS349 that had been grown in BG11 medium were incubated in $BG11_0$ medium (supplemented or not with an amino acid analog), they were observed not to grow (Fig. 2). Identical results were obtained with another mutant (strain CS387, not included in Table 3) which has amino acid transport characteristics similar to those of strains CS326 and CS349. Because the three mutants were isolated independently, the unselected phenotype of inability to grow on dinitrogen appears related to the phenotype of impairment in amino acid transport.

In contrast to mutants CS326 and CS349, mutant strains CS327, CS343, and CS389 were able to grow on dinitrogen, though their growth rates were lower than that of wild-type strain PCC 7120 (Fig. 2). Whereas growth on dinitrogen of strains CS327 and CS389 was observed either in the presence or in the absence of analogs (CS327, hydroxylysine; CS389, methionine sulfoximine), growth of strain CS343 on dinitrogen was observed only with no analog (azaleucine) added. Nevertheless, the dinitrogen-grown cultures of strain CS343 did not consist of an accumulation of revertants, as determined by measuring the uptake activity for some amino acids of the dinitrogen-grown cultures (data not shown). We are therefore confident that mutant CS343 is able to grow to some extent on dinitrogen, though the reason for its sensitivity to azaleucine in $BG11_0$ medium is unknown.

To test whether the amino acid transport mutants that were

TABLE 4. Accumulation of amino acids in the extracellular medium in cell suspensions of *Anabaena* sp. strain PCC 7120 and some amino acid analog-resistant mutants[a]

Medium	Amino acid accumulated	Concn (μM) of amino acid in the extracellular medium						
		PCC 7120	EF116	CS326	CS327	CS343	CS349	CS389
BG11	Ala	0.31	0.08	8.81	0.44	10.99	12.94	12.14
	Val	0.05	ND[b]	1.18	0.08	1.07	0.99	0.89
	Ile	0.05	ND	0.77	0.04	1.21	0.68	0.75
	Phe	0.03	ND	1.14	0.08	1.51	1.26	1.08
	Leu	0.07	ND	0.27	0.05	0.23	0.31	0.45
$BG11_0$	Ala	0.04	0.19	15.46	0.17	8.97	7.16	4.99
	Val	ND	0.03	1.00	ND	0.48	0.39	0.28
	Ile	ND	0.04	1.75	ND	1.73	1.06	0.66
	Phe	ND	0.04	2.61	ND	1.52	1.93	1.14
	Leu	ND	0.03	2.06	ND	1.35	0.94	0.74

[a] Cells grown on BG11 medium (supplemented with amino acid analogs in the case of the mutants; see Materials and Methods) were washed, resuspended in BG11 or $BG11_0$ medium, and incubated under culture conditions for 48 h. Samples of the cell suspensions were then filtered, and the filtrates were concentrated and analyzed by HPLC as described in Materials and Methods.

[b] ND, not detected.

Downloaded from http://jb.asm.org/ on July 25, 2017 by INSTITUTO DE PARASITOLOGFA Y

TABLE 5. Frequencies of heterocysts and nitrogenase activities in *Anabaena* sp. strain PCC 7120 and some amino acid analog-resistant mutants[a]

Strain	% Heterocysts	Nitrogenase (μmol/h/mg of Chl)
PCC 7120	5.3	7.1
CS326	<0.1	0.0
CS327	3.6	1.6
CS343[b,c]	<0.1	0.05
CS349[b]	0.5	0.0
CS389	3.0	1.6

[a] Cells grown on BG11 medium (with or without amino acid analogs; see Materials and Methods) were washed and resuspended in $BG11_0$ (combined nitrogen-free) medium. After 48 h of incubation under culture conditions, percentages of heterocysts and nitrogenase activity were determined. The data are the mean of three to eight determinations.

[b] Filaments were heavily fragmented (to a mean of fewer than two cells per filament) in the combined nitrogen-free medium.

[c] Higher nitrogenase levels, ca. 2 μmol/h/mg of Chl, were found when the cultures of strain CS343 were induced in $BG11_0$ medium for 72 h.

impaired in diazotrophic growth were able to develop dinitrogen-fixing heterocysts, induction experiments were performed. Mutant cells grown on BG11 medium were washed, resuspended in $BG11_0$ medium, and incubated under growth conditions for 48 h. Nitrogenase activity and frequency of heterocysts were then determined. None of the mutants was able to develop heterocysts or nitrogenase activity to the same extent as the wild-type strain PCC 7120 was (Table 5). Microscopic examination of the induced cell suspensions showed the presence of a low amount of heterocysts, rather than a normal percentage of immature heterocysts, in the mutants.

DISCUSSION

Amino acid transport systems. Results presented in this work show that the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120 exhibits a wide range of amino acid transport activities. Analysis of a number of amino acid transport mutants has shown, however, that those activities are carried out by only a small number of transport systems (Table 6 summarizes the results). Spontaneous mutants resistant to azaleucine were readily obtained by plating cells on analog-containing culture medium. Six of these mutants that were analyzed (data for one of which, strain CS343, are presented in Table 3) exhibited reduced levels of transport of neutral amino acids, notably of Pro, Phe, Gly, and Leu, whose uptake was reduced by ≥93% in these mutants. This finding suggests that strain CS343 lacks a transport system for neutral amino acids, which we shall call system N-I; this is the principal system for uptake of Pro, Phe, Gly, and Leu and is also an important system for uptake of Thr and Ala, in strain PCC 7120.

No spontaneous mutant of strain PCC 7120 resistant to azaserine or to methionine sulfoximine could be obtained in this work, which suggests that inactivation of more than one gene would be required to confer resistance of strain PCC 7120 to those analogs. This would be the case if those analogs could be taken up at significant rates by more than one transport system in this cyanobacterium. This interpretation is supported by the fact that spontaneous mutants resistant to azaserine or to methionine sulfoximine were readily obtained when mutant CS343 was used as a parental strain. Strain CS389 (isolated as a methionine sulfoximine-resistant derivative of CS343) showed negligible activities of uptake of neutral amino acids (with the exception of Gln) and therefore appears to have resulted from mutation in a second neutral amino acid transport system (system N-II). (However, the possibility that what we have called systems N-I and N-II were two separately mutable components of a single transport system cannot be ruled out.)

The two neutral amino acid transport systems defined by the analysis of mutants CS343 and CS389 show overlapping, though not identical, specificities. The broad specificities of these transport systems, as deduced from the range of amino acid uptake activities affected in the mutants, is corroborated by the observation that in general, the transport of neutral amino acids was inhibited by other neutral amino acids but much less inhibited by basic (His behaved to some extent as a neutral amino acid) and acidic amino acids (Table 2). Similarity between the K_s values determined for Ala, Asn, Gln, and Ser in mutant CS343 and the wild-type strain, together with the fact that single kinetic components were observed with the different amino acids in the wild-type strain, implies that the two transport systems for neutral amino acids would exhibit similar K_s values for the tested amino acids. Since uptake of neutral amino acids was concentrative and sensitive to DCCD and CCCP (Table 1), both systems for neutral amino acids appear to catalyze an active transport.

Some mutations appeared to simultaneously inactivate more than one transport system. Thus, the mutation in strain CS326 inactivated, in addition to the high-affinity system for basic amino acids (12), the two neutral amino acid transport systems, and the mutation in strain CS327 appears to have inactivated

TABLE 6. Amino acid transport systems of *Anabaena* sp. strain PCC 7120 as defined from amino acid analog-resistant mutants

Transport system[a]	Substrate amino acids[b]	Mutant (analog and parental strain)[c]
High-affinity system for basic amino acids	Arg, (His), Lys, (Orn)	CS326 (canavanine, PCC 7120) CS327 (canavanine, PCC 7120) CS349 (azaserine, CS343)
Neutral amino acid transport system N-I	(Ala), (Asn), (Gln), Gly, (His), Leu, (Met), (Orn), Phe, Pro, (Ser), (Thr)	CS343 (azaleucine, PCC 7120) CS326 (canavanine, PCC 7120)
Neutral amino acid transport system N-II	(Ala), (Asn), (Gln), (Met), (Ser), (Thr)	CS326 (canavanine, PCC 7120) CS327 (canavanine, PCC 7120) CS349 (azaserine, CS343) CS389 (methionine sulfoximine, CS343)

[a] In addition to the three systems here summarized, there appear to be at least two more amino acid uptake systems in strain PCC 7120: a low-affinity system for basic amino acids (12) and a system for the uptake of acidic amino acids (see text).

[b] Amino acids in parentheses appear to be transported by more than one of the systems summarized. Thus, mutation of a single system resulted only in a partial loss of the uptake of those amino acids (see data for strain CS343 in Table 3). The acidic amino acids (not included) appear to be transported to some extent by the two neutral amino acid transport systems.

[c] Strains that, as explained in the text, are suggested to have resulted from mutation in the indicated transport system(s). The amino acid analog and the parental strain used to isolate those mutant strains are indicated in parentheses.

Downloaded from http://jb.asm.org/ on July 25, 2017 by INSTITUTO DE PARASITOLOGFA Y

neutral amino acid transport system N-II and the high-affinity system for basic amino acids. Strain CS349 (isolated as an azaserine-resistant derivative of strain CS343) might have resulted from a mutation similar to that in strain CS327. The nature, whether structural or regulatory, of the gene(s) whose mutation simultaneously inactivate more than one transport system is currently unknown.

Anabaena sp. strain PCC 7120 exhibited low affinities for Asp and Glu. Some uptake of Asp and Glu may take place through the neutral amino acid transport systems, since strains CS343 and CS389, which we suggest to be affected in the neutral amino acid transport systems, show an impairment in the uptake of Asp and Glu. The uptake of neutral amino acids was only slightly inhibited by Asp and Glu (Table 2), suggesting that the neutral amino acid transport systems bear low affinities for the acidic amino acids. On the other hand, the uptake of Glu by strain PCC 7120 is inhibited by neutral amino acids (7). Asp and Glu may interact in their protonated (neutral) forms with the neutral amino acid transport systems. Transport of Asp and Glu through these active permeases may be responsible for the small fraction of uptake of these amino acids that is sensitive to DCCD and CCCP (Table 1). On the other hand, some other uncharacterized transport system(s) would be responsible for the uptake of Asp and Glu still observed in mutants CS326, CS349, and CS389. Transport catalyzed by such acidic amino acid transport system(s) might be passive in nature.

The differences observed between strains CS326, on one hand, and strains CS349 and CS389, on the other hand, in the uptake of acidic amino acids appear to be significant. The mutation in strain CS326 allowed higher levels of uptake of acidic amino acids than did the mutation in strain CS349 or strain CS389. In fact, strain CS326 was found to be sensitive to methionine sulfoximine whereas strains CS349 and CS389 were resistant to this analog, suggesting that methionine sulfoximine was also transported by the system(s) responsible for the uptake of acidic amino acids still operating in strain CS326.

Comparison of amino acid transport activities in mutants CS349 and CS389 suggests that Gln might be transported to some extent by the basic amino acid transport system. Transport of Gln through the basic amino acid permease has also been reported in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 (6, 16). On the other hand, His and Orn may be taken up to some extent by the neutral amino acid transport systems, as suggested by the behavior of mutant CS327, which still exhibited a high activity of uptake of His, and of mutant CS343, which was clearly affected in the uptake of Orn (Table 3).

To summarize, data currently available can be interpreted in terms of the presence of at least five amino acid transport systems in *Anabaena* sp. strain PCC 7120: three active transport systems, one rather specific for basic amino acids (12) and two with overlapping (though not identical) specificities for neutral amino acids, one passive system for basic amino acids (12), and one system for acidic amino acids that is yet to be characterized. At least the active transport systems appear to be expressed in cells grown on nitrate, dinitrogen, or ammonium.

The array of amino acid transport systems suggested in this work for *Anabaena* sp. strain PCC 7120 is similar, though not identical, to that proposed for *Synechocystis* sp. strain PCC 6803 (16). In strain PCC 6803, three amino acid transport systems have been described: one specific for basic amino acids and Gln, one specific for neutral amino acids (excluding Gln), and another one specific for Glu and Gln. Thus, the array of amino acid transport systems found in cyanobacteria is more similar to that thought to be prevalent in green algae and plants than to the one described for other bacteria like the enterobacteria or the pseudomonads (2). Whereas in green algae (25) and higher plants (17), a few transport systems with broad specificities have been found (i.e., one or two neutral amino acid transport systems, one system for basic amino acids, and one system for acidic amino acids), a large number of amino acid transport systems have been found in *Escherichia coli*, *Salmonella typhimurium*, and *Pseudomonas* spp. (i.e., systems specific for single amino acids or groups of chemically related amino acids, and more than one transport system for some particular amino acids or groups of chemically related amino acids). However, the structures of some of the cyanobacterial amino acid transport systems may be similar to those of the bacterial multicomponent (binding protein-dependent) transport systems (6).

Physiology of amino acid transport. A few amino acids, such as Arg, Asn, or Gln, can be used as sources of nitrogen by some cyanobacteria (5, 21), and therefore uptake of these amino acids to be used as nitrogen sources can be a physiological function of the corresponding amino acid transport systems.

An accumulation of some amino acids in the outer medium in *Anabaena* cell suspensions was observed in this work. The amino acids that accumulated in the culture medium most likely originated by leakage from healthy cells rather than from cell lysis. In the case of significant cell lysis, the amino acids found in the culture medium would be expected to correspond to those present intracellularly at the highest levels (i.e., Glu), but this was not the case. The accumulation of amino acids was higher in cell suspensions of some amino acid transport mutants than in cell suspensions of the wild-type strain (Fig. 1 and Table 4). This could reflect altered transport activity by a mutated permease resulting in a significant leak of some amino acids. Alternatively, a physiological function for some amino acid transport system(s) in *Anabaena* sp. strain PCC 7120 might be recapture of amino acids leaked out spontaneously from the cells, as has been suggested for some other permeases (1, 15). Some amino acids, in particular the hydrophobic amino acids, can leak out of the bacterial cell (15), and *Anabaena* sp. strain PCC 7120 released Ala as well as some other hydrophobic amino acids to the extracellular medium (Fig. 1 and Table 4). In *Anabaena* sp. strain PCC 7120, transport system N-I may be involved in recapture of amino acids, since mutants of this system accumulated in the outer medium some hydrophobic amino acids (namely, Ala, Val, Ile, Phe, and Leu) that are taken up mainly through this transport system (we assume that Val and Ile are transported by the same system as Leu). Consistently with this suggestion, strain CS327, which appears to be mutated in transport system N-II and in the high-affinity system for basic amino acids but not in transport system N-I, did not show any significant accumulation of amino acids in the culture medium. A set of amino acids similar to that released by the strain PCC 7120 mutants is also released by a mutant of *Synechocystis* sp. strain PCC 6803 impaired in the transport of neutral amino acids (16). In *Gloeothece* sp., some amino acid transport systems might also be involved in recapture of amino acids that are released during N_2 fixation in the dark and subsequently reassimilated in the light (8).

Some mutants of strain PCC 7120 that were severely impaired in amino acid transport were observed to be also impaired in diazotrophic growth. More precisely, the mutants did not show a normal percentage of heterocysts after 48 h of incubation in nitrogen-free medium (Table 5). Since extracellular accumulation of amino acids was observed in the presence as well as in the absence of combined nitrogen, impairment of growth on dinitrogen might be a consequence of the

Downloaded from http://jb.asm.org/ on July 25, 2017 by INSTITUTO DE PARASITOLOGFA Y

mutants being unable to cope with amino acid leakage when incubated in nitrogen-free medium. However, the behavior of mutant CS389 is not consistent with this simple hypothesis: this mutant grows on dinitrogen (Fig. 2) and develops heterocysts (Table 5) while releasing amino acids to a level similar to that of the mutants that were unable to grow on dinitrogen (Table 4).

It can be conjectured that in the *Anabaena* filament, some amino acid permeases may have a role in the intercellular transfer of metabolites that is essential both during heterocyst development upon nitrogen stepdown and during steady-state growth on dinitrogen (11, 33, 36). A mechanism for such a transfer might consist in the release of certain amino acids to the periplasmic space, where they might diffuse, perhaps bound to binding proteins, before being taken up (through amino acid permeases) by some adjacent cells. This hypothesis, which would imply the existence of a continuity in the periplasmic space along the filament, would explain why some amino acid transport systems are required for induction of diazotrophic growth in a heterocyst-forming cyanobacterium. The two mutants, strains CS326 and CS349, that were strongly hampered in growth on dinitrogen exhibited negligible activities of transport of basic and neutral amino acids (Table 3). Four amino acids, Gln, Glu, His, and Orn, were observed to be taken up at appreciable rates by every amino acid transport mutant that exhibited some ability to grow on dinitrogen (strains CS327, CS343, and CS389). Therefore, our results would be consistent with Gln (30) (and/or Glu, His, or Orn) having a key role in intercellular transfer of nitrogen in heterocyst-forming cyanobacteria. To further investigate the involvement of the transport systems identified in this work in diazotrophic growth, we are currently attempting the cloning of genes that encode proteins of those transport systems.

ACKNOWLEDGMENTS

This work was supported by grant PB90-0114 from Dirección General de Investigación Científica y Técnica and grant BIO93-0124 from Comisión Interministerial de Ciencia y Tecnología (Spain).

We thank E. Martínez-Force for useful advice with the HPLC.

REFERENCES

1. **Ames, G. F.-L.** 1988. Structure and mechanism of bacterial periplasmic transport systems. J. Bioenerget. Biomembr. **20:**1–18.
2. **Antonucci, T. K., and D. L. Oxender.** 1986. The molecular biology of amino-acid transport in bacteria. Adv. Microb. Physiol. **28:**145–180.
3. **Atfield, G. N., and C. J. O. R. Morris.** 1961. Analytical separations by high-voltage paper electrophoresis. Amino acids in protein hydrolysates. Biochem. J. **81:**606–614.
4. **Chapman, J. S., and J. C. Meeks.** 1983. Glutamine and glutamate transport by *Anabaena variabilis*. J. Bacteriol. **156:**122–129.
5. **Flores, E., and A. Herrero.** 1994. Assimilatory nitrogen metabolism and its regulation, p. 487–517. *In* D. A. Bryant (ed.), The molecular biology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
6. **Flores, E., and A. M. Muro-Pastor.** 1990. Mutational and kinetic analysis of basic amino acid transport in the cyanobacterium *Synechocystis* sp. PCC 6803. Arch. Microbiol. **154:**521–527.
7. **Flores, E., and M. I. Muro-Pastor.** 1988. Uptake of glutamine and glutamate by the dinitrogen-fixing cyanobacterium *Anabaena* sp. PCC7120. FEMS Microbiol. Lett. **56:**127–130.
8. **Flynn, K. J., and J. R. Gallon.** 1990. Changes in intracellular and extracellular α-amino acids in *Gloeothece* during N_2-fixation and following addition of ammonium. Arch. Microbiol. **153:**574–579.
9. **Fogg, G. E.** 1952. The production of extracellular nitrogenous substances by a blue-green alga. Proc. R. Soc. Lond. Ser. B **139:**372–397.
10. **Giddings, T. H., Jr., and L. A. Staehelin.** 1981. Observation of microplasmodesmata in both heterocyst-forming and non-heterocyst forming filamentous cyanobacteria by freeze-fracture electron microscopy. Arch. Microbiol. **129:**295–298.
11. **Haselkorn, R.** 1978. Heterocysts. Annu. Rev. Plant Physiol. **29:**319–344.
12. **Herrero, A., and E. Flores.** 1990. Transport of basic amino acids by the dinitrogen-fixing cyanobacterium *Anabaena* PCC 7120. J. Biol. Chem. **265:** 3931–3935.
13. **Ihlenfeldt, M. J. A., and J. Gibson.** 1975. CO_2 fixation and its regulation in *Anacystis nidulans* (*Synechococcus*). Arch. Microbiol. **102:**13–21.
14. **Jüttner, F.** 1983. ^{14}C-labeled metabolites in heterocysts and vegetative cells of *Anabaena cylindrica* filaments and their presumptive function as transport vehicles of organic carbon and nitrogen. J. Bacteriol. **155:**628–633.
15. **Krämer, R.** 1994. Secretion of amino acids by bacteria: physiology and mechanism. FEMS Microbiol. Rev. **13:**75–94.
16. **Labarre, J., P. Thuriaux, and F. Chauvat.** 1987. Genetic analysis of amino acid transport in the facultatively heterotrophic cyanobacterium *Synechocystis* sp. strain 6803. J. Bacteriol. **169:**4668–4673.
17. **Li, Z.-C., and D. R. Bush.** 1990. ΔpH-dependent amino acid transport into plasma membrane vesicles isolated from sugar beet leaves. Plant Physiol. **94:**268–277.
18. **Mackinney, G.** 1941. Absorption of light by chlorophyll solutions. J. Biol. Chem. **140:**315–322.
19. **Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87:**206–210.
20. **Martínez-Force, E., and T. Benítez.** 1991. Separation of o-phthalaldehyde derivatives of amino acids of the internal pool of yeast by reverse-phase liquid chromatography. Biotechnol. Tech. **5:**209–214.
21. **Neilson, A. H., and T. Larsson.** 1980. The utilization of organic nitrogen for growth of algae: physiological aspects. Physiol. Plant. **48:**542–553.
22. **Raboy, B., and E. Padan.** 1978. Active transport of glucose and α-methylglucoside in the cyanobacterium *Plectonema boryanum*. J. Biol. Chem. **253:** 3287–3291.
23. **Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman, and R. Y. Stanier.** 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. **111:**1–61.
24. **Rowell, P., S. Enticott, and W. D. P. Stewart.** 1977. Glutamine synthetase and nitrogenase activity in the blue-green alga *Anabaena cylindrica*. New Phytol. **79:**41–54.
25. **Sauer, N., and W. Tanner.** 1985. Selection and characterization of *Chlorella* mutants deficient in amino acid transport. Further evidence for three independent systems. Plant Physiol. **79:**760–764.
26. **Spence, D. W., and W. D. P. Stewart.** 1986. Proline inhibits N_2-fixation in *Anabaena* 7120. Biochem. Biophys. Res. Commun. **139:**940–946.
27. **Stewart, W. D. P.** 1963. Liberation of extracellular nitrogen by two nitrogen-fixing blue-green algae. Nature (London) **200:**1020–1021.
28. **Strasser, P., and G. Falkner.** 1986. Characterization of the glutamate/aspartate-transport system in a symbiotic *Nostoc* sp. Planta **168:**381–385.
29. **Thiel, T.** 1988. Transport of leucine in the cyanobacterium *Anabaena variabilis*. Arch. Microbiol. **149:**466–470.
30. **Thomas, J., J. C. Meeks, C. P. Wolk, P. W. Shaffer, S. M. Austin, and W.-S. Chien.** 1977. Formation of glutamine from [^{13}N]ammonia, [^{13}N]dinitrogen, and [^{14}C]glutamate by heterocysts isolated from *Anabaena cylindrica*. J. Bacteriol. **129:**1545–1555.
31. **Watanabe, A.** 1951. Production in cultural solution of some amino acids by the atmospheric nitrogen-fixing blue-green algae. Arch. Biochem. Biophys. **34:**50–55.
32. **Wolk, C. P.** 1968. Movement of carbon from vegetative cells to heterocysts in *Anabaena cylindrica*. J. Bacteriol. **96:**2138–2143.
33. **Wolk, C. P.** 1989. Alternative models for the development of the pattern of spaced heterocysts in *Anabaena* (*Cyanophyta*). Plant Syst. Evol. **164:**27–31.
34. **Wolk, C. P., S. M. Austin, J. Bortins, and A. Galonsky.** 1974. Autoradiographic localization of ^{13}N after fixation of ^{13}N-labeled nitrogen gas by a heterocyst-forming blue-green alga. J. Cell Biol. **61:**440–453.
35. **Wolk, C. P., Y. Cai, L. Cardemil, E. Flores, B. Hohn, M. Murry, G. Schmetterer, B. Schrautemeier, and R. Wilson.** 1988. Isolation and complementation of mutants of *Anabaena* sp. strain PCC 7120 unable to grow aerobically on dinitrogen. J. Bacteriol. **170:**1239–1244.
36. **Wolk, C. P., A. Ernst, and J. Elhai.** 1994. Heterocyst metabolism and development, p. 769–823. *In* D. A. Bryant (ed.), The molecular biology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
37. **Wolk, C. P., J. Thomas, P. W. Shaffer, S. Austin, and A. Galonsky.** 1976. Pathway of nitrogen metabolism after fixation of ^{13}N-labeled nitrogen gas by the cyanobacterium, *Anabaena cylindrica*. J. Biol. Chem. **251:**5027–5034.
38. **Xu, P., and P. J. McAuley.** 1990. Uptake of amino acids by the cyanobacterium *Anabaena* ATCC 27893. New Phytol. **115:**581–585.

Downloaded from http://jb.asm.org/ on July 25, 2017 by INSTITUTO DE PARASITOLOGFA Y