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Ammonia Assimilation in *Bacillus polymyxa*

^{15}N NMR AND ENZYMATIC STUDIES*

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Pathways of ammonia assimilation into glutamic acid and alanine in *Bacillus polymyxa* were investigated by ^{15}N NMR spectroscopy in combination with measurements of the specific activities of glutamate dehydrogenase, glutamine synthetase, glutamate synthase, alanine dehydrogenase, and glutamic-alanine transaminase. Ammonia was found to be assimilated into glutamic acid predominantly by NADPH-dependent glutamate dehydrogenase with a K_m of 2.9 mM for NH_4^+ not only in ammonia-grown cells but also in nitrate-grown and nitrogen-fixing cells in which the intracellular NH_4^+ concentrations were 11.2, 1.04, and 1.5 mM, respectively. In ammonia-grown cells, the specific activity of alanine dehydrogenase was higher than that of glutamic-alanine transaminase, but the glutamate dehydrogenase/glutamic-alanine transaminase pathway was found to be the major pathway of $^{15}NH_4^+$ assimilation into [^{15}N]alanine. The *in vitro* specific activities of glutamate dehydrogenase and glutamine synthetase, which represent the rates of synthesis of glutamic acid and glutamine, respectively, in the presence of enzyme-saturating concentrations of substrates and coenzymes are compared with the *in vivo* rates of biosynthesis of [^{15}N]glutamic acid and [α,γ-^{15}N]glutamine observed by NMR, and implications of the results for factors limiting the rates of their biosynthesis in ammonia- and nitrate-grown cells are discussed.

Glutamic acid and glutamine play key roles in the assimilation of ammonia by microorganisms. Ammonia can be incorporated into glutamic acid by the glutamate dehydrogenase (GDH) pathway:

$$NH_4^+ + \alpha\text{-ketoglutarate} + NAD(P)H + H^+ \overset{GDH}{\longleftrightarrow} \text{L-glutamate} + NAD(P)^+ + H_2O$$

or via glutamine by the glutamine synthetase(GS)/glutamate synthase (GOGAT) pathway:

$$NH_3 + \text{L-glutamate} + ATP \xrightarrow{GS} \text{L-glutamine} + ADP + P_i$$

$$\alpha\text{-Ketoglutarate} + \text{L-glutamine} + NAD(P)H + H^+ \xrightarrow{GOGAT} 2\ \text{L-glutamate} + NAD(P)^+.$$

In ammonia-rich medium, most microorganisms assimilate ammonia by the glutamate dehydrogenase pathway. When the supply of ammonia is limited, such as during growth in low concentrations of ammonia or upon nitrate or molecular nitrogen as a nitrogen source, assimilation by the glutamine synthetase/glutamate synthase pathway is more efficient because glutamine synthetase has a much lower K_m for NH_4^+ than does glutamate dehydrogenase (1). Thus, most microorganisms adapt to ammonia limitation by derepressing the glutamine synthetase/glutamate synthase pathway and repressing glutamate dehydrogenase (1–4). All N_2-fixing prokaryotes reported to date assimilate ammonia by the glutamine synthetase/glutamate synthase pathway during N_2 fixation (5, 6). A few microorganisms with low or undetectable levels of glutamate synthase derepress glutamate dehydrogenase in response to ammonia limitation (2, 7).

Among the species of *Bacillus*, spore-forming Gram-positive bacteria, some interesting anomalies have been found. Whereas *Bacillus licheniformis* (8) and *Bacillus megaterium* (4, 9, 10) utilize the glutamate dehydrogenase pathway in ammonia-rich medium and the glutamine synthetase/glutamate synthase pathway during ammonia limitation, *Bacillus subtilis* 168 lacks a biosynthetic NADP-glutamate dehydrogenase (11–13) and assimilates ammonia by the glutamine synthetase/glutamate synthase pathway even in ammonia-rich medium (14). This raises the question of how ammonia is assimilated in other species of *Bacillus*, particularly the N_2-fixing species such as *Bacillus polymyxa*, in which the pathways of ammonia assimilation have not been studied.

Another possible pathway of ammonia assimilation is via alanine, which can be synthesized directly from ammonia and pyruvate by alanine dehydrogenase (ADH):

$$NH_4^+ + \text{pyruvate} + NADH + H^+ \overset{ADH}{\longleftrightarrow} \text{L-alanine} + NAD^+ + H_2O$$

or by transamination from glutamic acid by glutamic-alanine transaminase (GAT):

$$\text{L-Glutamic acid} + \text{pyruvate} \overset{GAT}{\longleftrightarrow} \text{L-alanine} + \alpha\text{-ketoglutarate}$$

In the reversible alanine dehydrogenase reaction, the V_{max} for the biosynthesis of alanine greatly exceeds that for the catabolic reaction measured using purified alanine dehydrogenase from *B. subtilis* (15). The *Bacillus* species have much higher levels of alanine dehydrogenase than do other bacteria (16). Therefore, alanine dehydrogenase may play a role in the biosynthesis of alanine in *Bacillus* growing in ammonia-rich medium, although in cells grown with alanine as nitrogen or carbon source, alanine dehydrogenase, whose level is greatly induced, undoubtedly plays a catabolic role (9, 11, 17). No quantitative comparison to assess the relative contributions

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of the alternative pathways has been reported for the activities of alanine dehydrogenase and glutamic-alanine transaminase in ammonia-grown *Bacillus*.

To determine which of two alternative pathways of biosynthesis is the major pathway under a specific growth condition, the traditional method is to compare the specific activities of the enzymes catalyzing each pathway as well as their kinetic parameters such as the K_m for substrates. The major pathway can be identified by this method if the specific activities of the enzymes catalyzing the alternative pathways can be measured accurately and are found to be significantly different. However, an alternative method for determining the pathway is desirable if the enzymes are unstable or difficult to assay. If the specific activities of the enzymes (and their affinities for the substrates) of the alternative pathways are found to be comparable, a different approach is required to identify the major pathway.

The utility of ^{15}N NMR in studying metabolic processes in microorganisms (18–22) and plants (23, 24) is becoming increasingly evident, as has recently been reviewed (25–27). NMR has the advantage of permitting not only the identification of ^{15}N-metabolites through their characteristic resonance positions, but also quantitation through measurement of peak intensities in intact cells or cell extracts without need for separation from other metabolites. ^{15}N NMR provides a simple method of determining whether $^{15}NH_4^+$ is assimilated into [^{15}N]glutamic acid predominantly by the glutamine synthetase/glutamate synthase or the glutamate dehydrogenase pathway in *B. polymyxa* through observation of the time-dependent formation of ^{15}N-labeled glutamine and glutamic acid in cell-free extracts. An initial formation of [γ-^{15}N]glutamine followed by [^{15}N]glutamic acid indicates that the glutamine synthetase/glutamate synthase pathway is predominant. By contrast, an initial increase in [^{15}N]glutamic acid followed by [α,γ-^{15}N]glutamine indicates that the glutamate dehydrogenase pathway is predominant. We report here a study of the pathways of ammonia assimilation into glutamic acid and alanine in *B. polymyxa* by ^{15}N NMR in combination with measurements of the specific activities of the enzymes.

EXPERIMENTAL PROCEDURES

Strains, Media, and Growth—*B. polymyxa* ATCC 25901 (nonmucoid strain), ATCC 842, and ATCC 8519 were obtained from the American Type Culture Collection. For growth on ammonia (22 mM), nitrate (13 mM), L-glutamate (20 mM), or L-alanine (20 mM) as the nitrogen source, the growth medium, based on the medium of Katznelson and Lockhead (28), was prepared by mixing equal portions of the following two solutions after autoclaving: (*a*) $K_2HPO_4 \cdot 3H_2O$ (22.8 g), $MgSO_4$ (244 mg), $CaCl_2 \cdot 2H_2O$ (132 mg), NaCl (10 mg), $FeCl_2 \cdot 4H_2O$ (10 mg), $Na_2MoO_4 \cdot 2H_2O$ (5 mg), $MnCl_2 \cdot 4H_2O$ (8.8 mg), $ZnSO_4 \cdot 7H_2O$ (17.8 mg), NH_4Cl (1.177 g) or KNO_3 (1.31 g) or L-glutamic acid (2.94 g) or L-alanine (1.78 g), and distilled water (500 ml) (pH of the solution adjusted to 7.7); and (*b*) glucose (10.8 g), biotin (20 μg), and distilled water (500 ml). Growth was monitored in a Klett-Summerson colorimeter. Batch cultures (250–500 ml) were grown in baffled flasks on a shaker at 30 °C from an inoculum of 2–3 Klett units to midexponential phase (170 ± 15 Klett units).

For N_2 fixation, the nitrogen-free medium of Hino and Wilson (29) was used with the following modifications. $CaCl_2 \cdot 2H_2O$ (66 mg/liter) was substituted for $CaCO_3$, and the concentration of the $K_2HPO_4 \cdot KH_2PO_4$ buffer was 0.1 M. A 2.5% inoculum of ammonia-grown cells was added to the nitrogen-free medium (200 ml) in a 1-liter flask equipped with a Pyrex side arm for monitoring growth. The flask was closed with a rubber stopper fitted with glass tubes on which were placed short neoprene tubing closed with screw-type pinch clamps. Gas in the flask was evacuated with a vacuum pump to about 0.05 atm of pressure and refilled with high purity (99.99%) nitrogen. This was repeated three times; and after the fourth evacuation, the flask was filled with 0.8 atm of N_2 (the other 0.2 atm remaining as negative pressure to be replaced by fermentation gases). After every 30-Klett-Summerson unit increase in growth, the flask was evacuated of metabolic gases (CO_2 and H_2) and refilled with 0.8 atm of N_2. For large-scale cultures (250–500 ml), the cells were grown, after inoculation and evacuation of gas in 1- or 4-liter flasks with continuous flow of N_2 (200 ml/min) through a gas dispersion tube immersed in the culture.

NMR Experiments—Batch cultures of *B. polymyxa* grown to 170 ± 7 Klett units in medium containing ammonia or nitrate were collected by centrifugation and resuspended in a medium containing $^{15}NH_4Cl$ (22 mM) or $K^{15}NO_3$ (3 mM) and incubated at 30 °C on a shaker. At the times specified, a 500-ml aliquot of the culture was chilled rapidly to 5 °C (within 30 s) to terminate metabolic reactions, and cells were collected by centrifugation at 13,200 × *g* for 5 min at 4 °C. For the experiment requiring inhibition of glutamate dehydrogenase, the ammonia-grown cultures were transferred to, and incubated for 30 min in, nitrogen-free medium with (inhibited) or without (control) 0.3 M glutarate as the glutamate dehydrogenase inhibitor before the addition of $^{15}NH_4Cl$ to a final concentration of 22 mM.

Intracellular ^{15}N-metabolites were extracted with aqueous ethanol (30) to avoid degradation of heat-labile glutamine. The cell pellet was suspended in 60% ethanol (40 ml) and stirred vigorously for 1 h. The extraction was repeated three times. The cell pellet was then suspended in 0.2 N sodium citrate (5 ml) at pH 5 and incubated at 55 °C for 15 min for further extraction of polar amino acids. The pooled extracts were concentrated on a rotary evaporator at 40 °C to 10 ml, acidified to pH 2 to precipitate acid-insoluble materials, neutralized to pH 6.5, further concentrated, and transferred to a 10-mm (outer diameter) NMR sample tube after the addition of 10 mg of EDTA and 0.2 ml of ^2H_2O. Further extraction of the residual cell pellet and acid-insoluble material revealed that no ^{15}N-metabolites remained. Degradation of glutamine does not occur because the NMR resonance of [γ-^{15}N]glutamine, added to unlabeled cells in a separate experiment to test possible degradation, gave the same peak intensities before and after the extraction and concentration procedure.

The ^{15}N NMR spectra were obtained with a Bruker AM-500 spectrometer operating at 50.68 MHz. ^{15}N chemical shifts are reported in parts/million upfield from 1 M $H^{15}NO_3$. The operating conditions employed 20.5-μs (70° flip angle) pulse width, 7-s delays, and proton decoupling by WALTZ-16 composite pulse sequence. The delay time was found to allow 83% recovery of the equilibrium peak intensity for the γ-^{15}N of glutamine. Similar recovery is expected for the α-^{15}N of the amino acids studied here on the basis of our previous measurements (31) of their spin-lattice relaxation times (T_1). The sample temperature was 25 ± 2 °C.

For measurement of the rate of biosynthesis of ^{15}N-amino-acids, the quantities of biologically ^{15}N-labeled amino acids contained in each NMR sample of the cell-free extracts were calculated from their observed ^{15}N peak intensities by calibrating with standards as follows. ^{15}N peak intensities were measured under identical NMR operating conditions for 34.2 μmol each of [γ-^{15}N]glutamine, [^{15}N]glutamic acid, and [^{15}N]alanine dissolved in unlabeled cell extracts to ensure the same environments as that for the biologically ^{15}N-labeled amino acids. This ensures that the effect of T_1 on, and the contribution of nuclear Overhauser enhancement to, the peak intensities are the same for the respective ^{15}N nuclei of the standard and the biosynthesized amino acids. The normalized peak intensity/nanomole of ^{15}N-amino-acid of these standards was used to calculate the quantity (nanomoles) of each biologically ^{15}N-labeled amino acids in an NMR sample from its observed peak intensity. The quantity of ^{15}N-amino-acid in nanomoles was then divided by the amount of protein (milligrams) present in the cell from which the ^{15}N-amino-acids had been extracted (experimentally determined to be 1.47 μg of protein/Klett-Summerson unit) to obtain the nanomoles of ^{15}N-amino-acid/mg of protein.

Calculation of the Rate of Utilization of ^{15}N-Amino-acids—The average rate of utilization of [^{15}N]glutamic acid for protein synthesis in nitrate-grown cells during the first 10 min after transfer to $^{15}NO_3^-$ medium was estimated as follows. Nitrate-grown cells with a doubling time of 6.6 h (Table I) have a specific growth rate (μ) of 1.75×10^{-3} min^{-1} according to the formula: $\ln(X/X_0) = \mu t$, where X_0 and X are the biomass at time 0 and t, respectively. The percentage increase in the biomass (and therefore in cellular protein), $\Delta X = ((X - X_0)/X_0) \times 100$, is 1.8% after 10 min. On the assumption that glutamyl residues constitute 1/20th by weight of cellular proteins and that therefore there are 340 nmol of glutamyl residues/mg of protein, the amount of glutamic acid utilized for protein synthesis in 10 min of growth is $340 \times 0.018 = 6.12$ nmol/mg of protein. The percentage of [^{15}N]glutamic acid in the total free glutamic acid pool increases from 0 at time 0 to 37% at $t = 10$ min (Fig. 4*B*). Therefore, the average rate of utilization

of free [^{15}N]glutamic acid for protein synthesis over this time period is approximately $(6.12 \times 0.175)/10 = 0.11$ nmol of [^{15}N]glutamic acid·min^{-1}·mg^{-1} of protein.

Similarly, the average rate of utilization of [α,γ-^{15}N]glutamine for protein synthesis in ammonia-grown cells in the first 20 min after transfer to $^{15}NH_4^+$ medium can be calculated to be 0.4 nmol of [α,γ-^{15}N]glutamine·min^{-1}·mg^{-1} of protein. This figure is obtained from the doubling time of 3.2 h (Table I), which corresponds to $\mu = 3.6 \times 10^{-3}$ min^{-1} and $\Delta X = 7.5\%$ after 20 min, along with the percentage of free [α,γ-^{15}N]glutamine in the total glutamine pool (100 nmol/mg of protein), which increases from 0 at $t = 0$ to 65% at $t = 20$ min (Fig. 4*A*).

Enzyme Assays—All cells used for enzyme assays were grown to midexponential phase (170 ± 15 Klett units for ammonia- and nitrate-grown cells and 90 ± 5 Klett units for N_2-fixing cells), harvested by centrifugation at 13,200 × *g* for 5 min at 4 °C, and washed in the following buffers: 20 mM Tris-HCl (pH 7.5) containing 10 mM 2-mercaptoethanol for glutamate dehydrogenase and glutamate synthase; the same buffer supplemented with 10 mM $MnCl_2$ for glutamine synthetase; and 50 mM Tris-HCl (pH 8.0) buffer containing 1 mM 2-mercaptoethanol for alanine dehydrogenase and glutamic-alanine transaminase. The cells were sonicated 10 times for 30 s, with 30-s intervals for cooling, with a Fisher sonic dismembrator set at 35% power. The cell debris was removed by centrifugation at 17,200 × *g* for 10 min at 4 °C. The supernatant containing 5–20 mg of protein/ml was used for enzyme assays. All enzyme assays were performed at 20 °C within 30 min of harvesting the cells and were performed on duplicate cultures. Protein was measured by the method of Lowry *et al.* (32) with bovine serum albumin as standard. Unless otherwise indicated, the volume of each assay solution was 1.2 ml.

Glutamate dehydrogenase and glutamate synthase activities were determined spectrophotometrically by measuring the rate of oxidation of NADPH, and alanine dehydrogenase activity by measuring oxidation of NADH, at a wavelength of 340 nm by modifications of the standard procedures (4, 12, 15). The reaction mixture contained the following: for glutamate dehydrogenase assay, 50 mM Tris-HCl (pH 7.8), 5 mM 2-mercaptoethanol, 0.1 mM EDTA, 35 mM α-ketoglutarate, 80 mM NH_4Cl, and 0.3 mM NADPH; for glutamate synthase assay, 50 mM Hepes[1] buffer (pH 7.3), 5 mM α-ketoglutarate, 5 mM glutamine, and 0.3 mM NADPH; and for alanine dehydrogenase assay, 50 mM Tris-HCl (pH 8.0), 2 mM pyruvate, 100 mM NH_4Cl, and 0.24 mM NADH. Reactions were initiated by the addition of cell-free extracts, and the initial velocity was measured with a Hewlett-Packard 8450A diode array spectrophotometer. Under these conditions, the initial velocity was proportional to the enzyme concentration. Specific activities are reported as nanomoles of NADPH (glutamate dehydrogenase and glutamate synthase) or NADH (alanine dehydrogenase) oxidized per min/mg of protein. Low activities of NADPH or NADH oxidase, present in crude extracts, were determined with appropriate reagent blanks and were subtracted. Initial rates for the reverse reaction of glutamate dehydrogenase (oxidative deamination of L-glutamate) were determined by replacing α-ketoglutarate, NH_4Cl, and NADPH with 100 mM L-glutamate (Na^+) and 0.3 mM $NADP^+$. K_m values of glutamate dehydrogenase for ammonia and α-ketoglutarate and of alanine dehydrogenase for ammonia were determined by the method of Lineweaver and Burk (see Ref. 33).

Glutamic-alanine transaminase was assayed spectrophotometrically by method I of Segal *et al.* (34) modified as follows. The concentrations of L-alanine, NADH, and lactic dehydrogenase in the assay solution were 200 mM, 0.28 mM, and 1.67 units/ml, respectively.

Glutamine synthetase was assayed by a modification of the radiochemical method of Prusiner and Milner (35). The assay solution contained the following in a final volume of 1.1 ml: 50 mM MOPS buffer (pH 6.6), 5.5 mM NH_4Cl, 11.4 mM glutamic acid and 0.006 mM L-[U-^{14}C]glutamic acid (1.52 μCi/ml), 5.5 mM ATP, 5.5 mM $MnCl_2$, and 0.045 mM aminooxyacetate to inhibit pyridoxal phosphate-dependent glutamate decarboxylase activity. The reaction was initiated by addition of cell-free extracts and terminated at 1, 2, 3, 5, and 7 min by withdrawing a 220-μl aliquot of the reaction mixture into 1.4 ml of ice-cold water. The solution was immediately passed over a column (0.7 × 3 cm) of Dowex AG 1-Cl to separate [^{14}C]glutamine from [^{14}C]glutamic acid. The eluate that contains [^{14}C]glutamine was collected in a scintillation vial, and after the addition of 12 ml of Liquiscint (National Diagnostics, Inc.), the radioactivity was determined in a Beckman Model LS8100 scintillation counter. The low background radioactivity in the eluate due to unbound [^{14}C]glutamic acid (less than 0.5% of the total [^{14}C]glutamic acid added) was measured with the assay solution in the absence of cell-free extracts and was subtracted. On omission of ATP and Mn^{2+} from the reaction mixture, only the background radioactivity was observed. This result indicates that [γ-^{14}C]aminobutyrate (whose formation from [^{14}C]glutamic acid by glutamate decarboxylase in the cell-free extract would have given erroneously high radioactivity in the eluant (36)) was not formed under this assay condition.

Ammonia Assay—Intracellular NH_4^+ concentrations in NH_4^+-, NO_3^--, and N_2-grown cells during midexponential phase were determined on duplicate cultures by assaying for NH_4^+ by the phenol-hypochlorite method (37) after perchlorate extraction of NH_4^+ from the cell. This method has been shown to give an intracellular ammonium ion concentration within 3% of that determined by heat extraction of ammonia and its determination by an ammonia electrode (38). Cells from a 100-ml culture were collected by centrifugation and suspended in 0.3 N $HClO_4$ (2–7 ml) by stirring at 4 °C for 20 min. After removal of the acid-insoluble material by centrifugation, the supernatant was neutralized with KOH, and the precipitate of $KClO_4$ was removed by filtration. The NH_4^+ in the supernatant was determined by the phenol-hypochlorite method (37). Protein in the cell pellet was determined by suspending the acid-insoluble precipitates from the perchlorate solution in 1 N NaOH (7 ml) for 1 h at room temperature to solubilize protein and by assaying for protein by the method of Lowry *et al.* (32). The total ammonium ion and protein in the cell, obtained as described above, were used to calculate the nanomoles of NH_4^+/milligram of protein, which, in turn, was converted to intracellular NH_4^+ concentration (millimolar) by the method of Clark *et al.* (39), on the assumption that the composition of a bacterial cell by weight is 80% water, 10% protein, and 10% nonprotein cellular material.

In the measurement of intracellular NH_4^+ in NH_4^+-grown cells, correction was made for the amount of NH_4^+ that was contained in the medium trapped in the cell pellet after centrifugation. The volume of medium trapped in the cell pellet was determined by adding [*carboxyl*-^{14}C]carboxylinulin, a carbohydrate which is not taken up by the cells, to a 100-ml culture, collecting the cells by centrifugation, and measuring the radioactivity in the cell pellet relative to the total added radioactivity. The volume of medium trapped in the cell pellet was found to be 0.325 ± 0.060 ml/100 ml of culture. NH_4^+ contained in the trapped medium, calculated from the volume of trapped medium and the NH_4^+ concentration in the medium, was subtracted from the total NH_4^+ in the perchlorate extract in the calculation of the intracellular NH_4^+ concentration.

Chemicals—$^{15}NH_4Cl$ (99% ^{15}N), $K^{15}NO_3$ (98% ^{15}N), L-[^{15}N]glutamic acid (99% ^{15}N), and DL-[^{15}N]alanine (99% ^{15}N) were purchased from Cambridge Isotope Laboratories. L-[γ-^{15}N]Glutamine (95% ^{15}N) and L-[α-^{15}N]glutamine (99% ^{15}N) were purchased from MSD Isotopes. L-[U-^{14}C]Glutamic acid (250 mCi/mmol) and [*carboxyl*-^{14}C]carboxylinulin (1–3 μCi/mg) were purchased from ICN Biomedicals, Inc.

Dowex AG 1-X8-Cl (analytical grade, 200–400 mesh) was obtained from Bio-Rad. NADPH, NADH, and lactic dehydrogenase were purchased from Sigma. All other chemicals were reagent-grade.

RESULTS

Growth—Most strains of *B. polymyxa*, as the name *polymyxa* (much slime) implies, produce heavy slime consisting of highly viscous extracellular polysaccharides which are difficult to séparate from the cells and can interfere with the preparation of cell-free extracts and enzyme assays. A nonmucoid strain (ATCC 25901), isolated by Biswas *et al.* (40), is much easier to handle. Growth of the nonmucoid strain in 22 mM ammonia was compared with that of two wild-type strains, ATCC 842 and ATCC 8519, to investigate whether they differ in their ability to assimilate ammonia. The growth curves of the nonmucoid and the 842 strain (Fig. 1) are essentially identical, with a doubling time of 3.2 h. Furthermore, the nonmucoid and the wild-type 8519 strains have very similar doubling times in ammonia and nitrate as a nitrogen source (Table I). Therefore, it is reasonable to assume that the pathways of ammonia assimilation are identical

[1] The abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

in the nonmucoid and wild-type strains. The nonmucoid strain was used for all enzyme assays and NMR experiments with ammonia and nitrate as the nitrogen source, unless indicated otherwise.

Both the nonmucoid and 8519 strains were found to fix N_2 under strictly anaerobic conditions, but the protein yield was higher for the 8519 strain. This strain did not produce slime when grown with a continuous flow of N_2 (see "Experimental Procedures"). Therefore, this wild-type strain was used for enzyme assays with N_2 as the nitrogen source.

Nitrate taken up by the cell is converted by nitrate reductase to nitrite, which, in turn, is reduced by nitrite reductase to ammonia. N_2 is reduced to ammonia by the nitrogenase complex. Intracellular NH_4^+ concentrations of nitrate- and N_2-grown *B. polymyxa* were 1.04 ± 0.12 and 1.5 ± 0.4 mM, respectively (Table I), which, as expected, were significantly lower than those observed in ammonia-grown cells (11.2 ± 1.9 mM).

Pathway of Ammonia Assimilation into Glutamic Acid—The time sequence of biosynthesis of ^{15}N-amino-acids in ammonia-grown *B. polymyxa*, as observed by ^{15}N NMR spectra of the cell-free extracts at 10, 20, and 40 min after transfer to NH_4^+ medium, is shown in Fig. 2*A*. The ^{15}N peaks are assigned on the basis of our previous work (18, 19). Whereas the biosynthesis of [^{15}N]glutamic acid was very rapid with no increase in peak intensity after 10 min, the biosynthesis of [α,γ-^{15}N]glutamine was much slower, as shown by the gradual increase in the peak intensities of its amide ^{15}N (263.6 ppm) and its α-amino ^{15}N (334.93 ppm), which is resolved from the α-amino ^{15}N of glutamic acid (335.06 ppm) in the spectra at 20 and 40 min as shown in the expanded scale spectrum for the latter. A corresponding experiment in which the assimilation of $^{15}NH_4^+$ was terminated at shorter time intervals (Fig. 2*B*) showed that the glutamic acid pool was completely ^{15}N-labeled within 2 min. The observed sequence of formation, [^{15}N]glutamic acid followed by [α,γ-^{15}N]glutamine, clearly indicates that $^{15}NH_4^+$ is assimilated into glutamic acid predominantly by the glutamate dehydrogenase pathway and not by the glutamine synthetase/glutamate synthase pathway in ammonia-grown cells.

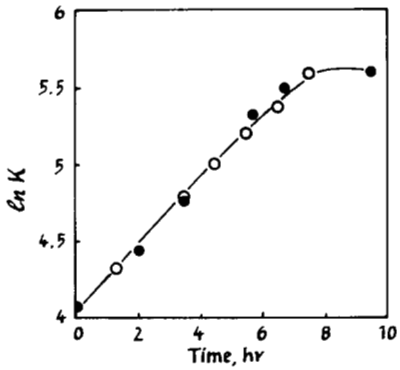

FIG. 1. **Growth of the nonmucoid ATCC 25901 (○) and wild-type ATCC 842 (●) strains of *B. polymyxa* in 22 mM NH_4^+ as nitrogen source at 30 °C.** *K*, cell density in Klett units. A single growth curve is shown because of the close similarity in the slopes (0.204 and 0.216) and the *y* intercepts (4.07 and 4.04) of the least-squares lines through the *open* and *closed circles*, respectively, during exponential growth.

Fig. 3 shows the biosynthesis of [^{15}N]glutamic acid and [γ-^{15}N]glutamine in nitrate-grown *B. polymyxa* as observed by ^{15}N NMR spectra of the cell-free extracts at 4, 10, and 60 min after transfer to $^{15}NO_3^-$ medium. After 4 min, $^{15}NH_4^+$, derived from $^{15}NO_3^-$, has been assimilated into [^{15}N]glutamic acid, but not to a detectable level into the amide ^{15}N of glutamine. Subsequently, [^{15}N]glutamic acid is formed much more rapidly than [γ-^{15}N]glutamine. The intracellular glutamine pool is large enough for the fully ^{15}N-labeled amide N of glutamine to be easily detectable by NMR (spectrum at 60 min). These results rule out the glutamine synthetase/glutamate synthase pathway as the major pathway of ammonia assimilation in nitrate-grown *B. polymyxa*. Assimilation of $^{15}NH_4^+$ by such a pathway would have resulted in the synthesis of a detectable level of [γ-^{15}N]glutamine prior to the formation of [^{15}N]glutamic acid; and after the glutamine pool had become fully ^{15}N-labeled, little or no further change in the [γ-^{15}N]glutamine peak intensity would have occurred. Therefore, the NMR results indicate that in nitrate-grown cells, glutamate dehydrogenase is the major pathway of ammonia assimilation.

Fig. 4 shows the time-dependent formations of the ^{15}N-amino-acids (in nanomoles/milligram of protein) calculated from their observed peak intensities (see "Experimental Procedures") in ammonia-grown (Fig. 4*A*) and nitrate-grown (Fig. 4*B*) cells. The rates of biosynthesis of some of these ^{15}N-amino-acids, calculated from their initial linear increase with time, are listed in Table I. For the biosynthesis of [^{15}N]glutamic acid in ammonia-grown cells, which was completed within 2 min after transfer to $^{15}NH_4^+$ medium (Fig. 2*B*), only the lower limit of the rate (36.4 nmol·min^{-1}·mg^{-1} of protein) could be calculated. [α,γ-^{15}N]Glutamine in ammonia-grown cells is synthesized at a rate of 3.3 nmol·min^{-1}·mg^{-1} of protein. In nitrate-grown cells, [^{15}N]glutamic acid is synthesized at a rate of 3.4 nmol·min^{-1}·mg^{-1} of protein. The nanomoles of [^{15}N]glutamic acid, [α,γ-^{15}N]glutamine, and [^{15}N]alanine/mg of protein at 60 min after the transfer to $^{15}NH_4^+$

TABLE I

Doubling times, metabolite pools, specific activities of ammonia-assimilating enzymes, and in vivo rates of biosynthesis of ^{15}N-amino-acids of B. polymyxa in different nitrogen sources

Nitrogen source	*B. polymyxa* strain	Doubling time	Intracellular concentration of metabolites				Specific activity					*In vivo* rate of biosynthesis		
			NH_4^+	Glu	Gln	Ala	GDH[a]	GS	GOGAT	ADH	GAT	Glu	Gln	Ala
		h	*mM*				*nmol·min^{-1}·mg^{-1} protein*					*nmol·min^{-1}·mg^{-1} protein*		
NH_4^+	Nonmucoid	3.2	11.2 ± 1.9	30	12.5	8	798 ± 186	23 ± 2	21 ± 5	35	19	>36.4	3.3	4.3
	8519	2.7					812							
NO_3^-	Nonmucoid	6.6	1.04 ± 0.12				932 ± 19	72 ± 10	17	11	30	3.4	<1.7	
	8519	6.1					471 ± 17							
Glutamate	Nonmucoid	6.5					287 ± 61							
Alanine	Nonmucoid	8.0								535				
N_2	8519	25	1.5 ± 0.4				313 ± 41	15 ± 2	12 ± 2					

[a] GDH, glutamate dehydrogenase; GS, glutamine synthetase; GOGAT, glutamate synthase; ADH, alanine dehydrogenase; GAT, glutamic-alanine transaminase.

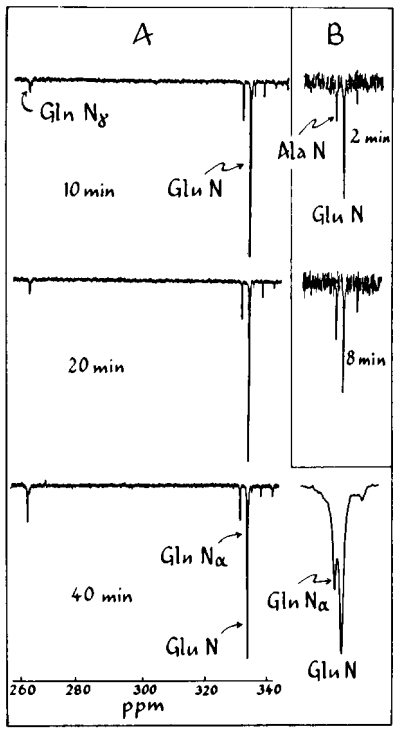

FIG. 2. **^{15}N NMR spectra of ^{15}N-amino-acids synthesized in ammonia-grown *B. polymyxa* cells on transfer to $^{15}NH_4^+$ medium and observed in the cell-free extracts after metabolic reactions were terminated at 10, 20, 40, and 60 min (*A*) and 2, 5, 8, 11, and 60 min (*B*).** Representative spectra from each experiment are shown in *A* and *B*, respectively. For the spectrum at 40 min (*A*), an expanded scale spectrum at the *right* shows resolved peaks for the α-^{15}N of glutamine and glutamic acid.

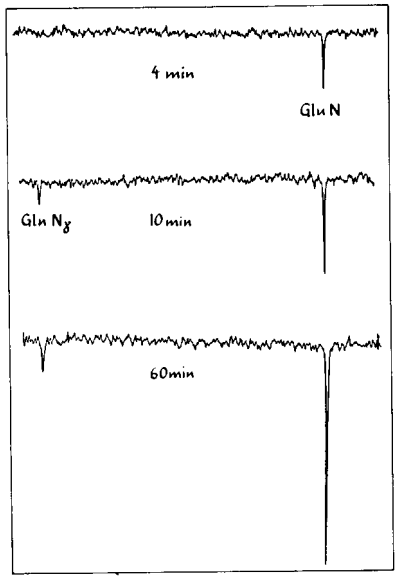

FIG. 3. **Representative ^{15}N NMR spectra of ^{15}N-amino-acids synthesized in nitrate-grown *B. polymyxa* on transfer to $^{15}NO_3^-$ medium and observed in the cell-free extracts.**

medium, when the intracellular pools of these amino acids had become fully ^{15}N-labeled (Figs. 2 and 4*A*), were used to calculate the intracellular concentrations of these amino acids in ammonia-grown cells by the method used to calculate the intracellular NH_4^+ concentration (see "Experimental Procedures"). The results are shown in Table I.

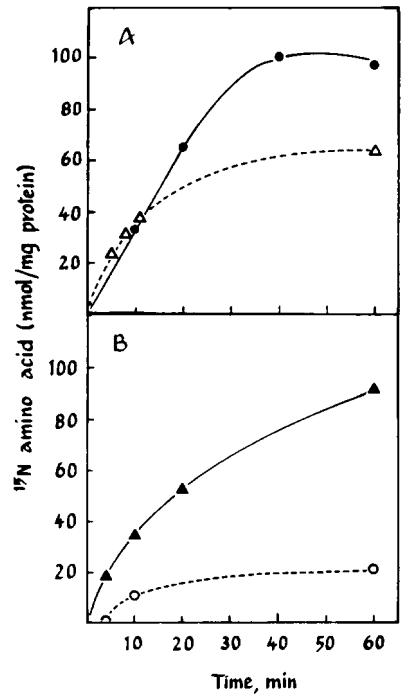

FIG. 4. **Time-dependent formation of ^{15}N-amino-acids in nanomoles/milligram of protein calculated from their observed ^{15}N peak intensities in ammonia-grown (*A*) and nitrate-grown (*B*) *B. polymyxa*.** *A*; ●, [α,γ-^{15}N]glutamine; △, [^{15}N] alanine; *B*: ○, [γ-^{15}N]glutamine; ▲, [^{15}N]glutamic acid.

Specific activities of glutamate dehydrogenase, glutamine synthetase, and glutamate synthase in ammonia-, nitrate-, and N_2-grown cells are shown in Table I. In ammonia-grown cells, the specific activity of glutamate dehydrogenase was more than 30-fold higher than that of glutamine synthetase or glutamate synthase. This result corroborates the result of the ^{15}N NMR experiment which showed that NH_4^+ is incorporated into glutamic acid predominantly by the glutamate dehydrogenase pathway in ammonia-grown cells. In nitrate-grown cells, where the NMR results indicated that $^{15}NH_4^+$ is assimilated directly into [^{15}N]glutamic acid by the glutamate dehydrogenase pathway, the specific activity of glutamate dehydrogenase was again very high relative to those of glutamate dehydrogenase and glutamate synthase, although glutamine synthetase is derepressed 3-fold in nitrate-grown cells relative to that in ammonia-grown cells. Surprisingly, in N_2-fixing cells, the activity of glutamate dehydrogenase is 20-fold higher than that of glutamine synthetase and glutamate synthase. This strongly suggests that *B. polymyxa*, unlike any N_2-fixing prokaryotes reported to date, assimilates ammonia mainly by the glutamate dehydrogenase pathway during N_2 fixation.

The specific activity of glutamate dehydrogenase of the ammonia-grown 8519 strain (812 nmol·min^{-1}·mg^{-1} of protein) is very close to that of the ammonia-grown nonmucoid strain. In the nitrate-grown 8519 strain which produced heavy slime, the specific activity of glutamate dehydrogenase (471 ± 17 nmol·min^{-1}·mg^{-1} of protein) was lower than that in the nonmucoid strain; it is likely that the presence of slime in the cell-free extracts interfered with the glutamate dehydrogenase assay. During N_2 fixation, the wild-type 8519 strain did not produce slime when grown with continuous flow of N_2. Therefore, the reported specific activities are considered to be reliable.

Coenzyme specificities of glutamate dehydrogenase and glutamate synthase were investigated, with the following re-

sults. Glutamate dehydrogenase of *B. polymyxa* is specific for NADPH; no NAD(H)-dependent glutamate dehydrogenase could be detected in the direction of glutamate formation or its deamination in either ammonia-grown cells or with L-glutamate as nitrogen source, a condition under which NAD^+-dependent glutamate dehydrogenase, if present, is expected to be maximally induced. Growth of *B. polymyxa* was not observed with glutamate as the sole source of carbon and nitrogen. For NADPH-glutamate dehydrogenase, the V_{max} for glutamate formation (287 ± 61 nmol·min^{-1}·mg^{-1} of protein in cell-free extracts of glutamate-grown cells) was approximately 5 times greater than the V_{max} for its deamination (54 nmol·min^{-1}·mg^{-1} of protein). Glutamate synthase of *B. polymyxa* is specific for NADPH; no NADH-dependent glutamate synthase could be detected. The activity of NADPH-glutamate synthase at pH 7.8 was no higher than at pH 7.3, the pH employed for the standard assay. The low specific activities of glutamate synthase are not due to the presence of glutaminase in the cell-free extracts. When a cell-free extract containing 2.9 mg of protein was added to a solution containing 5 mM glutamine in 50 mM Hepes buffer (pH 7.3), no NH_4^+ was produced after incubation for 10 min at 20 °C.

The glutamate dehydrogenase of *B. polymyxa* was found to have K_m values of 2.9 mM for NH_4^+ and 1.4 mM for α-ketoglutarate. A plot of $[1/V_0]$ versus $[1/NH_4^+]$ showed that at an NH_4^+ concentration of 1 mM, which prevails in nitrate-grown and N_2-fixing cells (Table I), the rate of assimilation of NH_4^+ by glutamate dehydrogenase at a given concentration of the enzyme is only one-fourth of the maximum rate. Despite the low affinity of glutamate dehydrogenase for NH_4^+, however, ammonia must be assimilated mainly by the glutamate dehydrogenase pathway in those ammonia-limited cells because the specific activities of glutamate dehydrogenase are 12- and 20-fold higher than those of glutamine synthetase in nitrate-grown and N_2-fixing cells, respectively.

Pathway of Alanine Biosynthesis—In ammonia-grown cells of *B. polymyxa*, alanine dehydrogenase was found to be present at a higher level (35 nmol·min^{-1}·mg^{-1} of protein) than glutamic-alanine transaminase (19 nmol·min^{-1}·mg^{-1} of protein) (Table I). Alanine dehydrogenase was found to have a K_m of 4.5 mM for NH_4^+ and is therefore capable of effectively assimilating NH_4^+ at an intracellular NH_4^+ concentration of 11.2 mM. Therefore, it is not clear from the specific activities of the enzymes whether alanine is synthesized directly from NH_4^+ and pyruvate by alanine dehydrogenase or via glutamic acid by glutamic-alanine transaminase in ammonia-grown cells. The ^{15}N NMR spectrum in Fig. 2*B*, which shows the time-dependent assimilation of $^{15}NH_4^+$ into [^{15}N]alanine and [^{15}N]glutamic acid, is compatible with either the direct incorporation of $^{15}NH_4^+$ into alanine by alanine dehydrogenase or the indirect incorporation via glutamic acid.

The pathway was determined by incubating the cells with $^{15}NH_4^+$ in the presence of glutarate, a glutamate dehydrogenase inhibitor (41), and observing the formation of [^{15}N]alanine and [^{15}N]glutamic acid by NMR. In *in vitro* assays, glutarate added at 0.05 M to the assay solution inhibited glutamate dehydrogenase of *B. polymyxa* by 64% and glutamic-alanine transaminase by less than 20%, but had no inhibitory effect on alanine dehydrogenase even at 0.5 M. Fig. 5 shows ^{15}N NMR spectra of ammonia-grown *B. polymyxa* incubated with $^{15}NH_4^+$ without (*A*) and with (*B*) a 30-min preincubation in 0.3 M glutarate. In the presence of the inhibitor, the synthesis of [^{15}N]glutamic acid decreased by 72% and the synthesis of [^{15}N]alanine decreased by 76%. A marked decrease was also observed in the synthesis of [^{15}N]aspartic acid (336.4 ppm), [^{15}N]valine (339.3 ppm), and [ϵ-^{15}N]lysine and/or [δ-^{15}N]ornithine (343 ppm), which are also formed by transamination from glutamic acid. By contrast, no change was observed for the amide ^{15}N of glutamine (263 ppm), which is formed by direct incorporation of $^{15}NH_4^+$ by glutamine synthetase and is not expected to be affected by inhibition of glutamate dehydrogenase provided that there is a sufficient pool of glutamic acid ([^{15}N]glutamic acid + unlabeled glutamic acid formed before the inhibition) to act as substrate. This result indicates that the major pathway of alanine biosynthesis is via glutamic acid by the glutamic-alanine transaminase in ammonia-grown cells despite the presence of a substantial level of alanine dehydrogenase. Therefore, the rate of biosynthesis of [^{15}N]alanine (4.3 nmol·min^{-1}·mg^{-1} of protein (Table I and Fig. 4*A*), observed by NMR) can be regarded as the *in vivo* glutamic-alanine transaminase activity in the direction of alanine formation. When *B. polymyxa* is grown with L-alanine as the sole nitrogen source, alanine dehydrogenase becomes strongly induced (Table I). Under this condition, alanine dehydrogenase obviously plays an important catabolic role to provide NH_4^+ for the synthesis of nitrogenous metabolites.

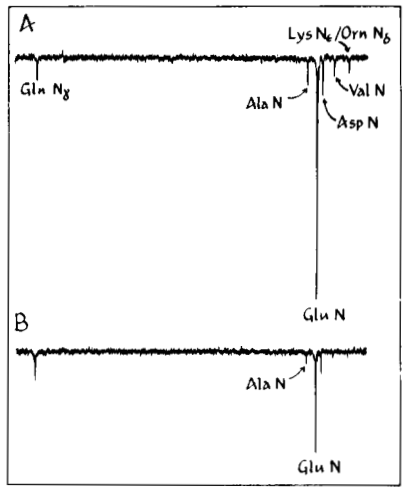

FIG. 5. **^{15}N NMR spectra of ^{15}N-amino-acids in *B. polymyxa* 10 min after addition of $^{15}NH_4^+$ without (*A*) and with (*B*) a 30-min preincubation in 0.3 M glutarate, a glutamate dehydrogenase inhibitor.**

DISCUSSION

Both the NMR experiments and the specific activities of glutamate dehydrogenase, glutamine synthetase, and glutamate synthase indicate that ammonia is assimilated into glutamic acid predominantly by the glutamate dehydrogenase pathway not only in ammonia-grown but also in nitrate-grown cells. In N_2-fixing *B. polymyxa*, the 20-fold higher specific activity of glutamate dehydrogenase relative to those of glutamine synthetase and glutamate synthase strongly suggests that glutamate dehydrogenase is the major pathway of ammonia assimilation under N_2 fixation. This is in marked contrast to all other N_2-fixing prokaryotes reported to date in which the glutamate synthase level is significantly higher (2, 5, 6) or at least comparable (42) to that of glutamate dehydrogenase during N_2 fixation. Therefore, the assimilation of ammonia by the glutamine synthetase/glutamate synthase pathway during N_2 fixation is not as ubiquitous a phenomenon as has previously been believed.

In *B. polymyxa*, the specific activity of glutamate synthase shows little variation with the nitrogen source, whereas the levels of glutamate dehydrogenase and glutamine synthetase appear to be regulated, as indicated by a 3-fold repression of glutamate dehydrogenase in glutamate-grown cells and a 3-

fold derepression of glutamine synthetase in nitrate-grown cells (Table I). Whether *B. polymyxa* possesses a mechanism for regulating glutamate synthase as do other species of *Bacillus* (4, 8) is not known at present.

In nitrate- and N_2-grown cells, where the intracellular NH_4^+ concentrations are about 1 mM, assimilation of ammonia by glutamate dehydrogenase with a K_m of 2.9 mM is expected to be inefficient. This must account, at least partially, for the observed slow growth of *B. polymyxa* in nitrate and N_2 (Table I) compared to other species that assimilate ammonia by the glutamine synthetase/glutamate synthase pathway. Nitrate-grown *B. polymyxa* has a doubling time of 6 h compared to 1.5 h for nitrate-grown *B. licheniformis* (8) at the same temperature. *B. polymyxa* fixes N_2 with a doubling time of 24 h compared to 2–3 h for *Clostridium pasteurianum* (43).

The inability to derepress the glutamine synthetase/glutamate synthase pathway in response to ammonia limitation is undoubtedly a disadvantage to *B. polymyxa* in its competition for survival with other N_2-fixing bacteria. Nevertheless, *Bacillus* and *Clostridium* are the most common free-living heterotrophic N_2-fixing bacteria found in forest soil, particularly in the rhizosphere of higher plants where a limited supply of combined nitrogen, low oxygen tension due to root respiration, and adequate supply of carbon and energy provide an environment suitable for N_2 fixation (44). *Bacillus* and *Clostridium* have an advantage over most other N_2-fixing bacteria in their ability to sporulate in response to nutrient limitation and to germinate when conditions become favorable for vegetative growth. Furthermore, *B. polymyxa* produces an antibiotic with a broad-spectrum bacteriocidal activity (45), polymyxin, which may kill or inhibit the growth of potential competitors in the natural habitat (46). *B. polymyxa* thrives because these advantages may offset the disadvantage of its apparent inability to adapt to nitrogen limitation through regulation of ammonia-assimilating enzymes.

^{15}N NMR is useful not only in distinguishing between alternative pathways of biosynthesis such as glutamate dehydrogenase *versus* glutamine synthetase/glutamate synthase or glutamate dehydrogenase/glutamic-alanine transaminase *versus* alanine dehydrogenase but also in providing a means to estimate the rates of biosynthesis of some important nitrogenous metabolites. The rates of biosynthesis of glutamic acid and glutamine can be estimated from the observed initial rates of biosynthesis of [^{15}N]glutamic acid and [α,γ-^{15}N]glutamine in Fig. 4 provided that (i) on transfer of the cells to ^{15}N-labeled medium, the intracellular NH_4^+ pool is ^{15}N-labeled at a rate that is rapid relative to the rates of biosynthesis of these amino acids, and (ii) during the period in which the formation of [^{15}N]glutamic acid or [α,γ-^{15}N]glutamine is apparently linear with time, the conversion of [^{15}N]glutamic acid or [α,γ-^{15}N]glutamine to other metabolites and proteins is negligible. The ^{15}N labeling of the intracellular NH_4^+ pool in ammonia-grown cells was completed in less than 2 min (time at which the glutamic acid pool was fully ^{15}N-labeled, Fig. 2*B*) and is therefore rapid relative to the observed rate of formation of [α,γ-^{15}N]glutamine. In nitrate-grown cells, the ^{15}N labeling of the low intracellular NH_4^+ pool can reasonably be expected to be rapid relative to the observed rates of formation of [^{15}N]glutamic acid and [γ-^{15}N]glutamine. The conversion of [^{15}N]glutamic acid to other metabolites in nitrate-grown cells in the first 10 min after transfer to $^{15}NO_3^-$ medium (*i.e.* the period of linear increase from which the rate of its biosynthesis was calculated) is expected to be negligibly small because (*a*) formation of [^{15}N]alanine or other ^{15}N-amino-acids as a result of transamination from [^{15}N]glutamic acid cannot be observed (Fig. 3), and (*b*) the average rate of utilization of [^{15}N]glutamic acid for protein synthesis during this period is estimated to be 0.11 nmol of [^{15}N]glutamic acid·min^{-1}·mg^{-1} of protein (see "Experimental Procedures"), which is very small compared to the observed rate of biosynthesis of [^{15}N]glutamic acid (3.4 nmol·min^{-1}·mg^{-1} of protein). Similarly, conversion of [α,γ-^{15}N]glutamine to other cellular constituents in ammonia-grown cells during the first 20 min after transfer to $^{15}NH_4^+$ medium is expected to be small on the basis of the following considerations. The γ-nitrogen of glutamine is utilized for the biosynthesis of the ω-nitrogen of arginine, the π-nitrogen of histidine, and the indole nitrogen of tryptophan, as well as for some nucleotides; but the steady-state concentrations of these metabolites are low. The concentration of arginine, for example, which is the most abundant among these, is only 40% of that of glutamine according to determination of the amino acid pool by high pressure liquid chromatography (data not shown). The absence of ^{15}N peaks corresponding to these nitrogenous metabolites in the ^{15}N spectra also suggests that their concentrations are low relative to that of [α,γ-^{15}N]glutamine. The average rate of utilization of [α,γ-^{15}N]glutamine for protein synthesis during this period is estimated to be 0.4 nmol·min^{-1}·mg^{-1} of protein (see "Experimental Procedures"), which, although not negligible, is small relative to the observed rate of [α,γ-^{15}N]glutamine biosynthesis (3.3 nmol·min^{-1}·mg^{-1} of protein). These considerations suggest that the rates of biosynthesis of the ^{15}N-amino-acids observed by NMR represent reasonable estimates of the actual rates of biosynthesis in the cell.

It is informative to compare the *in vivo* rates of biosynthesis of glutamic acid and glutamine (in nanomoles·$minute^{-1}$·$milligram^{-1}$ of protein) in the cell with the *in vitro* specific activities of glutamate dehydrogenase and glutamine synthetase which measure the rates of synthesis of glutamic acid and glutamine, respectively (in nanomoles·$minute^{-1}$·$milligram^{-1}$ of protein), when all substrates and coenzymes are present at enzyme-saturating concentrations, after taking into account the difference in temperature (30 °C for the measurement of *in vivo* rates and 20 °C for the enzyme assays). Such comparisons, when combined with the observed intracellular concentrations of some of the substrates and potential inhibitors, give us insight into the factors that are likely to be limiting the rates of biosynthesis of these amino acids in the cell. In ammonia-grown cells, the *in vivo* rate of biosynthesis of glutamine is substantially lower than the optimal rate expected from the *in vitro* glutamine synthetase activity (Table I). Kinetic properties of glutamine synthetase of *B. polymyxa* are not known at present, but purified glutamine synthetase from other *Bacillus* species are remarkably similar, with K_m values of 0.3–0.4 mM for NH_4^+, 0.8–3.6 mM for glutamic acid, and 0.2–0.9 mM for Mn·ATP (or Mg·ATP) (47–49). In the presence of Mn^{2+}, *in vitro* glutamine synthetase activity is optimum when the ATP:manganese ratio is 1:1 and decreases sharply with any deviation from this ratio; while in the presence of Mg^{2+}, the activity is maximum when the ATP:magnesium ratio is in excess of 4:1 (47, 48). The glutamine synthetase activity is inhibited by AMP, glutamine, or alanine (49, 50), with 50% inhibition at 5 mM glutamine or alanine (48). For glutamine biosynthesis in ammonia-grown *B. polymyxa*, the observed intracellular concentrations of the substrates, NH_4^+ (11.2 mM) and glutamic acid (30 mM) (Table I), appear to be sufficient for optimal rate of glutamine biosynthesis, but the observed *in vivo* rate is less than one-seventh of the optimal rate expected from the *in vitro* glutamine synthetase activity. The most likely factors limiting the biosynthetic rate are nonoptimal physiological levels of ATP and Mn^{2+} (or Mg^{2+}) and partial inhibition of the energy-

requiring glutamine synthetase activity by glutamine and alanine which are present in the cell at 12.5 and 8 mM, respectively (Table I). In nitrate-grown *B. polymyxa*, the *in vitro* rate of biosynthesis of glutamic acid is less than 1/300th of the optimal rate expected from the *in vitro* glutamate dehydrogenase activity. Whereas the poor availability of the substrate NH_4^+ (1 mM) and the low affinity of glutamate dehydrogenase for NH_4^+ (K_m = 2.9 mM) must clearly be limiting the *in vivo* rate, the markedly low rate suggests that there are additional limiting factors. In nitrate-grown cells, the availability of NADPH for glutamate biosynthesis may well be limited due to requirement for reduced coenzymes by assimilatory nitrate and nitrite reductases.

The demonstrated utility of ^{15}N NMR for measuring the *in vivo* rates of synthesis of amino acids, when combined with measurements of the intracellular concentrations of all the substrates, coenzymes, potential inhibitors and activators of the enzymes involved, as well as their specific activities, will open the way to a clearer understanding of the actual factor(s) controlling the rates in the cell.

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