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Glutamate Biosynthesis in *Bacillus azotofixans*

^{15}N NMR AND ENZYMATIC STUDIES*

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Pathways of ammonia assimilation into glutamic acid in *Bacillus azotofixans*, a recently characterized nitrogen-fixing species of *Bacillus*, were investigated through observation by NMR spectroscopy of *in vivo* incorporation of ^{15}N into glutamine and glutamic acid in the absence and presence of inhibitors of ammonia-assimilating enzymes, in combination with measurements of the specific activities of glutamate dehydrogenase, glutamine synthetase, glutamate synthase, and alanine dehydrogenase. In ammonia-grown cells, both the glutamine synthetase/glutamate synthase and the glutamate dehydrogenase pathways contribute to the assimilation of ammonia into glutamic acid. In nitrate-grown and nitrogen-fixing cells, the glutamine synthetase/glutamate synthase pathway was found to be predominant. NADPH-dependent glutamate dehydrogenase activity was detectable at low levels only in ammonia-grown and glutamate-grown cells. Thus, *B. azotofixans* differs from *Bacillus polymyxa* and *Bacillus macerans*, but resembles other N_2-fixing prokaryotes studied previously, as to the pathway of ammonia assimilation during ammonia limitation. Implications of the results for an emerging pattern of ammonia assimilation by alternative pathways among nitrogen-fixing prokaryotes are discussed, as well as the utility of ^{15}N NMR for measuring *in vivo* glutamate synthase activity in the cell.

Our recent studies of the pathways of ammonia assimilation into glutamic acid in N_2-fixing *Bacillus* showed that the glutamate dehydrogenase (GDH) pathway:

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

is the predominant pathway in N_2-fixing cells of *Bacillus polymyxa* (1) and a major pathway in those of *Bacillus macerans* (2). This is in marked contrast to all other N_2-fixing prokaryotes studied previously which have been shown to assimilate ammonia by the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway:

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

$$\alpha\text{-ketoglutarate} + \text{L-glutamine} + NAD(P)H + H^+ \overset{GOGAT}{\longrightarrow} 2\ \text{L-glutamate} + NAD(P)^+$$

The coupled pathway is efficient for assimilating ammonia at low concentrations by virtue of the low K_m of glutamine synthetase for ammonia (3). *B. polymyxa* and *B. macerans* have glutamate dehydrogenases with K_m for NH_4^+ of 2.9 and 2.2 mM, respectively, whereas all other N_2-fixing prokaryotes studied previously (4) have either a glutamate dehydrogenase with unusually high K_m (>11 mM) for NH_4^+ or barely detectable levels of glutamate dehydrogenase even in ammonia-rich media (see Ref. 2). These findings raised the possibility that, for prokaryotes having a glutamate dehydrogenase with K_m for NH_4^+ in the common range of 1–5 mM, the glutamate dehydrogenase pathway which does not consume ATP may be more advantageous than the ATP-requiring glutamine synthetase/glutamate synthase pathway during the energy-demanding process of N_2 fixation, particularly for anaerobic N_2 fixers that must generate ATP through the inefficient process of fermentation. By contrast, N_2-fixing prokaryotes lacking glutamate dehydrogenase or having a glutamate dehydrogenase with a very high K_m for NH_4^+ must by necessity assimilate ammonia by the glutamine synthetase/glutamate synthase pathway.

Recently, a new N_2-fixing *Bacillus* species, *B. azotofixans*, has been isolated from Brazilian soil and characterized (5). *B. azotofixans* is identical with the *Bacillus* species that had been isolated by Hino and Wilson (6) and tentatively classified as the Hino strain of *B. polymyxa* although it differed from other *B. polymyxa* strains in its inability to ferment lactose, arabinose, and glycerol. *B. azotofixans* and the Hino strain have now been conclusively shown to be a separate species from *B. polymyxa* and to grow on N_2 much more efficiently than *B. polymyxa* or *B. macerans* (5). This raises the possibility that the mode of assimilation of ammonia derived from N_2 may be quite different in *B. azotofixans* and merits investigation in view of the unusual characteristics observed for the other two N_2-fixing *Bacillus* species.

^{15}N nuclear magnetic resonance (NMR) spectroscopy is useful for determining whether $^{15}NH_4^+$ is assimilated into glutamic acid by the glutamate dehydrogenase or the glutamine synthetase/glutamate synthase pathway through observation of time-dependent assimilation of ^{15}N into γ-N of glutamine and glutamic acid N in cells incubated with ^{15}N-labeled precursor (1, 7). It also permits measurement of *in vivo* rates of biosynthesis of these amino acids. This paper reports a study of the pathways of ammonia assimilation in ammonia-, nitrate-, and N_2-grown *B. azotofixans* by ^{15}N NMR in combination with measurements of the specific activities of ammonia-assimilating enzymes.

EXPERIMENTAL PROCEDURES

Strains, Media, and Growth—B. azotofixans ATCC 35681, *B. polymyxa* ATCC 8519, and *B. macerans* ATCC 8515 were obtained

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from the American Type Culture Collection.

For growth on combined nitrogen sources, the nitrogen-free medium described previously (1) was modified by substituting D-mannitol (60 mM) for D-glucose (60 mM) to reduce slime and supplementing with NH_4Cl (22 mM), KNO_3 (20 mM), L-alanine (20 mM), or L-glutamate (20 mM) as the nitrogen source. Batch cultures were grown aerobically on a shaker from an inoculum of 2–3 Klett units to midexponential phase at 30 °C unless specified otherwise.

For N_2 fixation, the nitrogen-free medium of Hino and Wilson (6) was used with D-mannitol (60 mM) substituted for sucrose to reduce slime, $CaCl_2 \cdot 2H_2O$ (66 mg/liter) for $CaCO_3$, and 0.1 M $K_2HPO_4 \cdot KH_2PO_4$ buffer. A 2.5% inoculum of ammonia-grown cells was added to the nitrogen-free medium (2 liters) in a 4-liter flask. After evacuation of gas from the flask, the culture was grown with a continuous flow of N_2 (200 ml/min) at 30 °C as described previously (1).

NMR Experiments—For study of the biosynthesis of [^{15}N]amino acids, batch cultures of *B. azotofixans* grown to 120 Klett/ml in NH_4Cl or 160 Klett/ml in KNO_3 at 21 ± 1 °C were collected by centrifugation and resuspended in a medium containing $^{15}NH_4Cl$ (16 mM) or $K^{15}NO_3$ (7.5 mM) for further growth. At specified times, a 250-ml aliquot of the culture was chilled rapidly to 5 °C (within 1 min) to terminate metabolic reactions, and cells were collected by centrifugation at 13,200 × *g* for 5 min at 4 °C.

For experiments involving inhibition of ammonia-assimilating enzymes, ammonia- or nitrate-grown cells were transferred to, and incubated for 20 min at 30 °C in, nitrogen-free medium without (control) or with (inhibited) 0.3 M glutarate (glutamate dehydrogenase inhibitor), 22 mM DL-methionine DL-sulfoximine or 11 mM L-methionine DL-sulfoximine (glutamine synthetase inhibitor), 0.5 mM azaserine (glutamate synthase inhibitor), or 4 mM aminooxyacetate (transaminase inhibitor) before the addition of $^{15}NH_4Cl$ (16 mM) or $K^{15}NO_3$ (7.5 mM) and incubation for 30 min.

The intracellular ^{15}N-metabolites were extracted with aqueous ethanol and concentrated for NMR experiments as described previously (1). For measurement of the rate of biosynthesis of [^{15}N]amino acids, the nanomoles of each biologically ^{15}N-labeled amino acid in an NMR sample was calculated from its observed peak intensity (in area), and then divided by the amount of protein (milligrams) present in the cell (experimentally determined to be 1.26 μg of protein/Klett unit) to obtain the nanomoles of [^{15}N]amino acid · mg^{-1} of protein, as described previously (1). Intracellular concentration of an [^{15}N]amino acid was calculated from the nanomoles of [^{15}N]amino acid · mg^{-1} of protein in cells which had been grown with 22 mM $^{15}NH_4Cl$ from an inoculum of 2–3 Klett units to midexponential phase.

The proton-decoupled ^{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 per million (ppm) upfield from 1 M $H^{15}NO_3$. The operating conditions were as described previously (1).

Enzyme Assays—The cell-free extracts for enzyme assays were prepared by harvesting cells at midexponential phase (116 ± 4 Klett/ml for ammonia- and glutamate-grown cells, 120 ± 25 Klett/ml for alanine-grown cells, 160 ± 2 Klett/ml for nitrate-grown cells, 180 ± 13 Klett/ml for N_2-fixing cells), washing with the specified buffers and disrupting the cells by sonication as described previously (1). All enzyme assays were performed at 21 ± 1 °C within 1 h of harvesting the cells. Protein was measured by the method of Lowry *et al.* (8).

Glutamate dehydrogenase, glutamate synthase, and alanine dehydrogenase activities were determined spectrophotometrically by modifications of the standard procedures (9–11) as described previously (1) with the following exception. The glutamate dehydrogenase assay solution contained 5 mM α-ketoglutarate, 80 mM NH_4Cl, and 0.3 mM NADPH in 50 mM $KH_2PO_4 \cdot K_2HPO_4$ buffer, pH 7.8. Specific activities are reported as milliunits, *i.e.* nmol of NADPH (glutamate dehydrogenase and glutamate synthase) or NADH (alanine dehydrogenase) oxidized per minute, per milligram of protein. In the cell-free extracts of N_2-fixing cells, where the oxidation of NADPH (at 0.3 mM concentration) by NADPH oxidase and other enzymes in the extracts was too rapid for accurate measurement of glutamate-synthase activity, the activity was assayed by measuring the rate of formation of [^{15}N]glutamate from [γ-^{15}N]glutamine by ^{15}N NMR spectroscopy as described previously (2). NADH-dependent glutamate dehydrogenase activity in ammonia-grown cells, where the NADH oxidase activity was high, was measured by addition of cell-free extracts to an assay solution containing 50 mM $KH_2PO_4 \cdot K_2HPO_4$ buffer, pH 7.8, 5 mM α-ketoglutarate, 80 mM $^{15}NH_4Cl$, and 17.5 mM NADH. At 11, 20, and 40 min, the reaction was terminated by withdrawing a 2-ml aliquot of the reaction mixture and acidifying to pH 2.0. The extent of formation of [^{15}N]glutamic acid was measured by ^{15}N NMR.

Glutamine-synthetase activity was measured by a modification of the radiochemical method of Prusiner and Milner (12) as described previously (1). The K_m value of alanine dehydrogenase for NH_4^+ was determined by the method of Lineweaver and Burk (see Ref. 13).

The intracellular NH_4^+ concentration in N_2-fixing cells was determined on a duplicate culture as described previously (1). For ammonia-grown cells, the intracellular NH_4^+ concentration could not be determined because the amount of NH_4^+ trapped in the residual slime in the unwashed cell pellet was large relative to intracellular NH_4^+.

Chemicals—$^{15}NH_4Cl$ (99% ^{15}N) and $K^{15}NO_3$ (98% ^{15}N) were purchased from Cambridge Isotope Laboratories, and L-[γ-^{15}N]glutamine (95% ^{15}N) from MSD Isotopes. All other chemicals were reagent grade.

RESULTS

Growth—Table I shows the doubling times of *B. azotofixans* in various nitrogen and carbon sources. *B. azotofixans*, when grown with D-glucose as the carbon source, produced heavy slime consisting of viscous extracellular polysaccharides which are difficult to separate from the cells. Slime production was effectively reduced when D-mannitol was used as a carbon source (14). *B. azotofixans* grew with very similar doubling times when D-mannitol was substituted for D-glucose in am-

TABLE I

Doubling times, specific activities of ammonia-assimilating enzymes, and in vivo rates of biosynthesis of [^{15}N] glutamic acid in B. azotofixans in different nitrogen sources

Nitrogen source	Carbon source	Doubling time		Intracellular concn. of metabolites			Specific activity[a]				*In vivo* rate[a] Glu
		30 °C	21 °C	NH_4^+	Glu	Gln	GDH[b]	GS	GOGAT[c]	ADH	
		h		*mM*			*milliunits · mg^{-1} protein*				
NH_4^+	Mannitol	2.8	5.2		63	20	6.8 ± 0.4	18.4	16.8[d]	186 ± 68	12.5
	Glucose	2.7									
NO_3^-	Mannitol	4.4	8.3		>30[e]	11[e]	ND[f]	29.1	15.4[d]		11.7
Glu	Mannitol	8.1					4.6		≤2.7[g]		
Ala	Mannitol	7.5								3272 ± 472	
N_2	Mannitol	9.1		0.4			ND	38.0	11.4[g]		
	Sucrose	9.8									

[a] At 21 ± 1 °C.
[b] GDH, glutamate dehydrogenase; GS, glutamine synthetase; GOGAT, glutamate synthase; ADH, alanine dehydrogenase.
[c] NADPH-dependent activity.
[d] Measured spectrophotometrically.
[e] Calculated from the peak intensities in the 30-min spectra in Fig. 1*A*.
[f] ND, not detectable.
[g] Measured by ^{15}N NMR.

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monia-grown cells or for sucrose in N_2-fixing cells (Table I). Thus, its growth is not limited when D-mannitol is the carbon source. Substitution of D-mannitol for D-glucose was found to have no significant effect on the specific activities of glutamate dehydrogenase in ammonia-grown *B. polymyxa* ATCC 8519 (990 milliunits·mg^{-1} protein (mannitol) *versus* 798 milliunits·mg^{-1} protein (glucose)) or in *B. macerans* ATCC 8515 (195 milliunits·mg^{-1} protein (mannitol) *versus* 276 milliunits·mg^{-1} protein (glucose)). Thus, it is reasonable to assume that the substitution of D-mannitol for D-glucose has no effect on the pathways of ammonia assimilation in the *Bacillus* species. D-mannitol was used as the carbon source throughout this study.

In ammonia-grown cells, the initial NH_4^+ concentration in the medium was 22 mM; increasing the concentration to 60 or 100 mM slowed the growth to doubling times of 4.6 and 6.6 h, respectively, at 30 °C. *B. azotofixans*, although characterized as nitrate-reductase negative (5) on the complex media of Gordon *et al.* (15), was found to grow aerobically with nitrate as the sole nitrogen source as described earlier for the Hino strain (6). The intracellular NH_4^+ concentration in N_2-fixing cells was found to be 0.4 ± 0.18 mM. Although the intracellular NH_4^+ concentration in nitrate- and ammonia-grown *B. azotofixans* could not be determined due to residual slime in the cell pellet, it is reasonable to assume that the cells are growing under ammonia-limited and ammonia-rich conditions, respectively, as was found for *B. polymyxa* (1).

Ammonia Assimilation in Nitrate- and N_2-grown Cells—

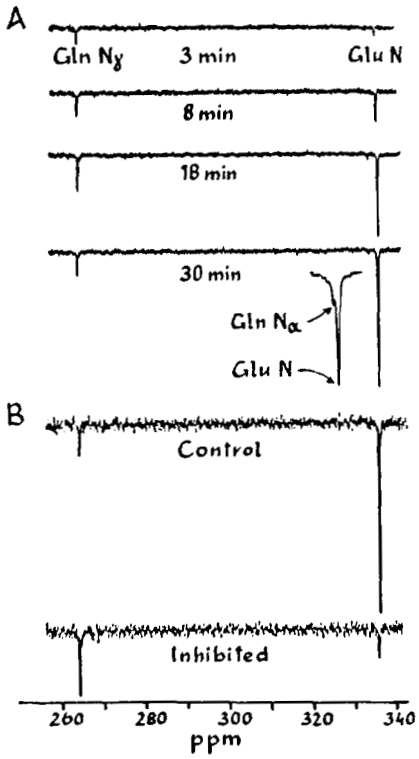

FIG. 1. **^{15}N NMR spectra of ^{15}N amino acids synthesized in (*A*) nitrate-grown *B. azotofixans* cells at 21 ± 1 °C on transfer to $^{15}NO_3^-$ (7.5 mM) medium and observed in the cell-free extracts after metabolic reactions were terminated at 3, 8, 18, and 30 min (for the spectrum at 30 min, an expanded-scale spectrum shows resolved peaks for the α-^{15}N of glutamine and glutamic acid); *B*, nitrate-grown cells at 30 °C preincubated without (control) or with (inhibited) 22 mM DL-methionine DL-sulfoximine + 0.5 mM azaserine for 20 min before the addition of K$^{15}NO_3$ (7.5 mM) and incubation for 30 min.**

Fig. 1*A* shows the biosynthesis of [α-γ-^{15}N]glutamine and [^{15}N]glutamic acid in nitrate-grown *B. azotofixans* as observed by NMR at 3, 8, 18, and 30 min after transfer to $^{15}NO_3^-$ medium. The ^{15}N peaks were assigned on the basis of previous work (1, 16). The $^{15}NO_3^-$, taken up by the cell, is converted by nitrate reductase to $^{15}NO_2^-$ which, in turn, is reduced by nitrite reductase to $^{15}NH_4^+$. After 3 min, as a result of $^{15}NH_4^+$ assimilation, 54–68% of the glutamine pool was ^{15}N-labeled in the γ-N (peak at 263.6 ppm) whereas only 6% of the glutamic acid was ^{15}N-labeled relative to the [^{15}N]glutamic acid pool observed after 30 min. The observed ±12% variation in the [γ-^{15}N]glutamine peak intensities between the 18- and 30-min spectra probably arises from experimental error in the extraction of ^{15}N metabolites. The average of the two peak intensities was taken to represent fully ^{15}N-labeled glutamine γ-N, because in a similar experiment performed at 30 °C (instead of 21 ± 1 °C) where metabolic processes are expected to proceed approximately twice as fast, [γ-^{15}N]glutamine peak intensities were found to show no increase between 8, 18, and 30 min. The glutamic acid pool, on the other hand, gradually becomes ^{15}N-labeled over a period of 30 min (peak at 335.06 ppm). By 18–30 min, a part of the [^{15}N]glutamic acid pool had been recycled by the glutamine-synthetase reaction to form [α,γ-^{15}N]glutamine whose α-^{15}N peak (334.93 ppm) was resolved from the α-amino ^{15}N peak of glutamic acid as shown in the expanded-scale spectrum. The extensive ^{15}N-labeling of the glutamine γ-N prior to that of the glutamic acid N strongly suggests that $^{15}NH_4^+$ is assimilated mainly by the glutamine synthetase/glutamate synthase pathway in nitrate-grown cells.

Fig. 1*B* shows the effect of inhibitors of glutamine synthetase and glutamate synthase on the biosynthesis of [α,γ-^{15}N]glutamine and [^{15}N]glutamic acid in nitrate-grown cells. L-Methionine DL-sulfoximine and azaserine (a structural analog of glutamine) are irreversible inhibitors of glutamine synthetase and glutamate synthase, respectively, but have no inhibitory effect on glutamate dehydrogenase (17–19). Preincubation of cells with 22 mM DL-methionine DL-sulfoximine + 0.5 mM azaserine prior to the addition of $^{15}NO_3^-$ resulted in 93% inhibition of the biosynthesis of [^{15}N]glutamic acid as indicated by the decrease in its peak intensity compared to that in the control (Fig. 1*B*). DL-Methionine DL-sulfoximine was added at the high concentration because at 4 mM concentration (in the absence of azaserine), $^{15}NH_4^+$ incorporation into the γ-N of glutamine was inhibited by only 12%. The extensive inhibition observed in the presence of the two inhibitors clearly shows that glutamic acid is formed predominantly by the glutamine synthetase/glutamate synthase pathway in nitrate-grown cells.

Fig. 2 shows the time-dependent formation of [^{15}N]glutamic acid (in nmol·mg^{-1} protein) calculated from the observed peak intensities in Fig. 1*A*. The nmoles of [^{15}N]glutamic acid·mg^{-1} protein formed at 18 and 30 min were calculated from the combined peak intensities of α-amino nitrogens of glutamic acid and glutamine because the latter is recycled from the former. The rate of biosynthesis of [^{15}N]glutamic acid calculated from Fig. 2 is 11.7 ± 0.3 nmol·min^{-1}·mg^{-1} protein (Table I). The average rate of utilization of [^{15}N]glutamic acid for protein synthesis during the 30-min interval, calculated from the doubling time at 21 ± 1 °C (Table I) by the method described previously (1) is less than 0.2 nmol [^{15}N]glutamic acid·min^{-1}·mg^{-1} protein which rate is negligibly small compared with the observed rate of its biosynthesis. Because the study with the inhibitors has shown that glutamate is biosynthesized predominantly by the glutamine synthetase/glutamine synthase pathway, the rate of [^{15}N]glutamic acid biosynthesis, when its substrate glutamine has become fully ^{15}N-

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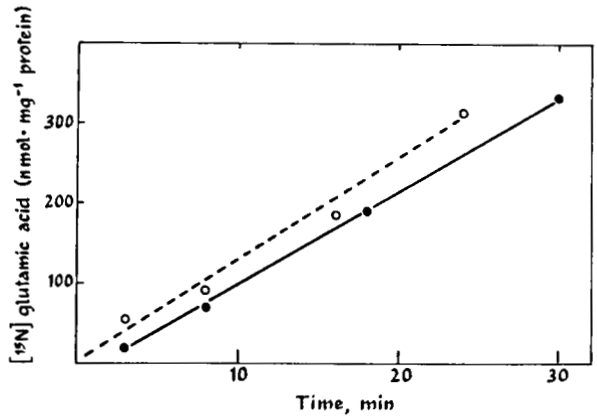

FIG. 2. **Time-dependent formation of [^{15}N]glutamic acid in nmol•mg^{-1} protein in nitrate-grown (●) and ammonia-grown (○) cells.**

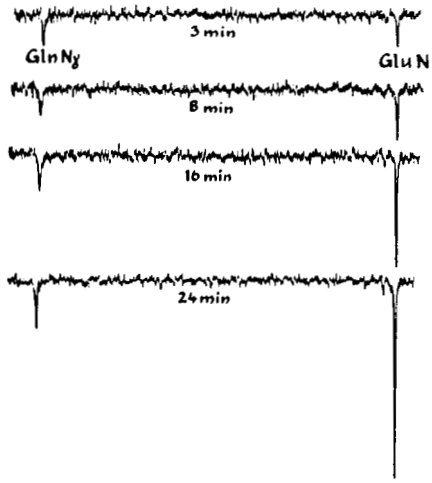

FIG. 3. **^{15}N NMR spectra of ^{15}N-amino acids synthesized in ammonia-grown *B. azotofixans* cells at 21 ± 1 °C on transfer to $^{15}NH_4^+$ (16 mM) medium and observed in the cell-free extracts after metabolic reactions were terminated at 3, 8, 16, and 24 min.**

labeled in the γ-N, can be regarded as the *in vivo* activity of glutamate synthase in the cell.

The specific activities of the ammonia-assimilating enzymes in nitrate-grown cells are listed in Table I. Glutamate dehydrogenase activity was undetectable, whereas glutamine synthetase and glutamate synthase activities were 29.1 and 15.4 milliunits·mg^{-1} protein, respectively. The result corroborates NMR studies and shows that the glutamine synthetase/glutamate synthase pathway is the predominant pathway of ammonia assimilation in nitrate-grown *B. azotofixans*. The *in vitro* specific activity of glutamate synthase, 15.4 milliunits·mg^{-1} protein, is reasonably close to the *in vivo* activity, 11.7 milliunits·mg^{-1} protein observed by NMR. This shows that the enzyme is stable under the assay conditions and that the *in vivo* activity is not substantially limited, compared to the *in vitro* activity, by intracellular concentrations of substrates. This is understandable because the intracellular concentration of glutamine is 11 mM (Table I) and the glutamate synthase of *B. azotofixans* is expected to have low K_m values (<0.2 mM) for its substrates as do the glutamate synthases of other *Bacillus* species (see "Discussion").

In N_2-fixing cells, the glutamate dehydrogenase activity was undetectable whereas glutamine synthetase and glutamate synthase activities were 38 and 11.4 milliunits·mg^{-1} protein, respectively. The result strongly suggests that in N_2-fixing cells of *B. azotofixans*, ammonia is assimilated predominantly by the glutamine synthetase/glutamate synthase pathway.

Ammonia Assimilation in Ammonia-grown Cells—The time-dependent incorporation of $^{15}NH_4^+$ into glutamine and glutamic acid in ammonia-grown cells, as observed by NMR at 3, 8, 16, and 24 min after transfer to $^{15}NH_4^+$ medium, is shown in Fig. 3. At 3 min, approximately 70% of the glutamine pool has been ^{15}N-labeled in the γ-N, whereas for glutamic acid, only 18% was ^{15}N-labeled relative to the pool at 24 min. The extensive ^{15}N-labeling of glutamine γ-N, combined with the slightly faster ^{15}N-labeling of glutamic acid compared to that in nitrate-grown cells (Fig. 1*A*) suggests that while ammonia may be assimilated mainly by the glutamine synthetase/glutamate synthase pathway, direct assimilation by the glutamate dehydrogenase pathway also contributes to the formation of [^{15}N]glutamic acid. The result rules out glutamate dehydrogenase as the predominant pathway because, in such case, the glutamic acid pool would have been rapidly saturated with ^{15}N, prior to the glutamine pool, as observed for ammonia-grown *B. polymyxa* (1). The rate of biosynthesis of [^{15}N]glutamic acid in ammonia-grown cells was found to be 12.5 ± 0.9 nmol·min^{-1}·mg^{-1} protein (Fig. 2 and Table I).

The specific activities of ammonia-assimilating enzymes are shown in Table I. Ammonia-grown cells had glutamate dehydrogenase activity of 5.0 milliunits·mg^{-1} protein at 60 Klett/ml and 6.8 ± 0.4 milliunits·mg^{-1} protein at 112 Klett/ml but had no detectable activity at 170 Klett/ml. The K_m of glutamate dehydrogenase for NH_4^+ could not be determined because of the low activity. The ammonia-grown cells had glutamine synthetase activity of 18.4 milliunits·mg^{-1} protein and glutamate synthase activity of 16.8 milliunits·mg^{-1} protein, as well as alanine dehydrogenase activity of 186 ± 68 milliunits·mg^{-1} protein (Table I). The results suggest that, while assimilation occurs through the glutamine synthetase/glutamate synthase pathway and to a lesser extent through the glutamate dehydrogenase pathway, assimilation via alanine by the alanine dehydrogenase/alanine-glutamic transaminase pathway may also occur in view of the observed high level of alanine dehydrogenase relative to other ammonia-assimilating enzymes.

To investigate the relative contributions of the three pathways to glutamate biosynthesis, the incorporation of $^{15}NH_4^+$ into glutamic acid and alanine was studied in the presence of the following inhibitors: L-methionine DL-sulfoximine (glutamine synthetase inhibitor) + azaserine (glutamate synthase inhibitor), aminooxyacetate (alanine-glutamic transaminase inhibitor (20)), and glutarate (an inhibitor of glutamate dehydrogenase (21) and, to a lesser extent, of glutamate synthase). Through *in vitro* assays, glutarate, a structural analog of α-ketoglutarate, added at 0.05 M concentration to the assay solution was shown to inhibit glutamate dehydrogenase by 64% and glutamic-alanine transaminase by less than 20% in *B. polymyxa* (1), and glutamate synthase by 36% in *B. azotofixans*.

Fig. 4 shows ^{15}N NMR spectra of ammonia-grown *B. azotofixans* incubated with $^{15}NH_4^+$ without (*A*) or with (*B*) 20 min of preincubation in 11 mM L-methionine DL-sulfoximine + 0.5 mM azaserine. In the presence of the inhibitors, the synthesis of [^{15}N]glutamic acid decreased by 30–40%. The inhibition of [^{15}N]glutamic acid formation was calculated from the ratio of the peak intensity in the inhibited cells to that in the control cells using the combined peak intensities (in integrated areas as shown in the expanded-scale spectra) for the α-amino ^{15}N of glutamic acid and that of glutamine because the latter is recycled from the former. The result is consistent with a substantial contribution of the glutamine

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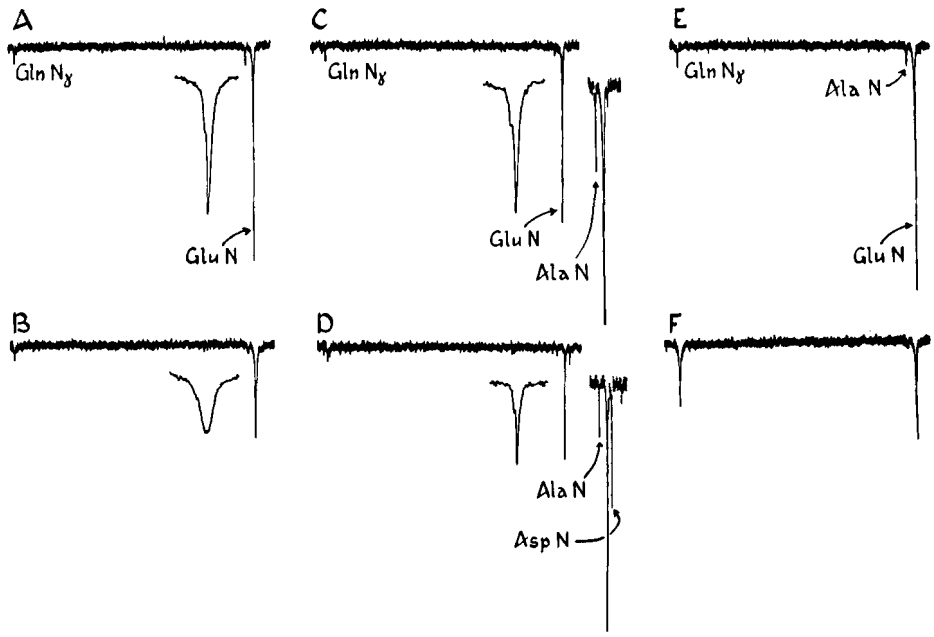

FIG. 4. **^{15}N NMR spectra of ammonia-grown *B. azotofixans* (30 °C) preincubated for 20 min without (*A*, *C*, and *E*) or with 11 mM L-methionine DL-sulfoximine + 0.5 mM azaserine (*B*), 0.3 M glutarate (*D*), or 4 mM aminooxyacetate (*F*) before the addition of $^{15}NH_4^+$ (16 mM) and incubation for 30 min.** Each spectrum in *C–F* represents an accumulation of 300 scans. *Insets* to *C* and *D* show ^{15}N spectra of the cells in *C* and *D* obtained after 3600 scans for comparison of alanine peak intensities.

synthetase/glutamate synthase pathway to the assimilation of NH_4^+. Preincubation with 0.5 mM azaserine alone resulted in 8–13% inhibition of [^{15}N]glutamic acid formation (result not shown).

Fig. 4*C* and *D* show the effect of preincubation with 0.3 M glutarate. In the presence of the inhibitor, the synthesis of [^{15}N]glutamic acid decreased by 59%. The result is consistent with participation of the glutamate dehydrogenase pathway to ammonia assimilation. The more extensive inhibition by glutarate than by L-methionine DL-sulfoximine + azaserine is understandable because glutarate has inhibitory effects on both glutamate dehydrogenase and glutamate synthase.

In the presence of aminooxyacetate, which inhibits the reversible glutamic-alanine transaminase as well as other transaminases (20), the biosynthesis of [^{15}N]alanine decreased to a barely detectable level (Fig. 3*E* (control) *versus* Fig. 3*F* (inhibited)). This suggests that [^{15}N]alanine is synthesized predominantly via [^{15}N]glutamic acid by transamination and not directly from $^{15}NH_4^+$ and pyruvate by alanine dehydrogenase. In the latter case, an increase in [^{15}N]alanine would occur in the inhibited cells due to inhibition of transamination to [^{15}N]glutamic acid. (The observed decrease in [^{15}N]glutamic acid synthesis in the inhibited cells compared to the control is probably due to accumulation of unlabeled glutamic acid during preincubation with aminooxyacetate which slowed down the glutamate dehydrogenase-catalyzed assimilation of $^{15}NH_4^+$ and channeled it into the glutamine-synthetase pathway.) Further evidence supporting this pathway for alanine biosynthesis is the 53% decrease in [^{15}N]alanine biosynthesis, comparable to the 59% decrease in [^{15}N]glutamic acid biosynthesis, observed in cells incubated with glutarate (Fig. 3, *C* and *D*, *insets*). Thus, the alanine dehydrogenase/alanine-glutamic transaminase pathway makes little, if any, contribution to $^{15}NH_4^+$ assimilation into [^{15}N]glutamic acid, despite the observed high level of alanine dehydrogenase. Alanine dehydrogenase was found to have an apparent K_m of 7.6–18 mM for NH_4^+; a more precise determination was precluded by nonlinearity of the Lineweaver-Burk plot of $1/v_o$ *versus* $1/[NH_4^+]$. The low affinity for NH_4^+ probably precludes participation of alanine dehydrogenase in ammonia assimilation. The 18-fold induction of alanine dehydrogenase observed in alanine-grown cells (Table I) suggests that its physiological function is mainly catabolic.

In cells incubated with glutarate, formation of [^{15}N]aspartic acid (peak at 336.4 ppm in Fig. 4*D*, *inset*) increased substantially compared to the control. The cause of this increase is unknown at present, but direct incorporation of $^{15}NH_4^+$ into aspartic acid by the aspartate dehydrogenase-catalyzed reaction with oxaloacetate or by the aspartase-catalyzed reaction with fumarate is unlikely because (i) the occurrence of aspartate dehydrogenase has not been confirmed in bacteria (22), (ii) succinate dehydrogenase, which catalyzes the synthesis of fumarate, is lacking in the Hino strain (23), and (iii) in the cells incubated with aminooxyacetate, an accumulation of [^{15}N]aspartic acid due to inhibition of transamination to [^{15}N]glutamic acid which is expected to occur if either one of the direct pathways were operative, is not observed (Fig. 4, *E* and *F*).

Properties and Regulation of the Enzymes—Coenzyme specificities of glutamate dehydrogenase and glutamate synthase were investigated with the following results. Only NADPH-dependent glutamate dehydrogenase activities were detected in *B. azotofixans*. No NADH-dependent glutamate dehydrogenase could be detected in ammonia-grown cells by spectrophotometric or NMR methods ("Experimental Procedures") or in glutamate-grown cells in which NAD^+-dependent glutamate dehydrogenase, if present, is expected to be maximally induced. The coenzyme specificity of glutamate synthase was investigated through observation of the time-dependent formation of [^{15}N]glutamic acid from [γ-^{15}N]glutamine with NADPH (Fig. 5*A*) or NADH (Fig. 5*B*) on addition of cell-free extracts of N_2-fixing *B. azotofixans*. NADPH- and NADH-dependent activities of 11.4 and 7.8 ± 3.4 milliunits·mg^{-1} protein, respectively, were observed. The results suggest that the glutamate synthase of *B. azotofixans* can utilize NADPH or NADH. In this respect, the glutamate synthase of *B. azotofixans* appears to resemble that of *B. subtilis* PCI 219 which has been shown to utilize NADPH or NADH with relative activities of 100 and 21, respectively (24).

Glutamine synthetase is derepressed 1.5-fold in nitrate-grown cells and 2-fold in N_2-fixing cells relative to ammonia-grown cells (Table I). Glutamate synthase activities show little variation with the nitrogen source except in glutamate-grown cells. Fig. 5 shows the result of glutamate synthase assays in which the time-dependent formation of [^{15}N]glutamate from [γ-^{15}N]glutamine was compared for N_2-fixing

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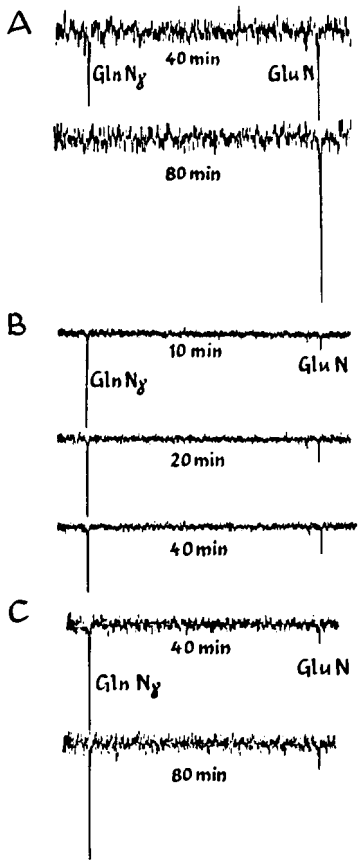

FIG. 5. **Glutamate synthase assay for nitrogen-fixing cells with NADPH (*A*) or NADH (*B*) as coenzyme, and for glutamate-grown cells with NADPH (*C*).** Cell-free extracts (12.5 mg protein/2 ml assay solution) were added to a solution containing 5 mM [γ-^{15}N]glutamine, 5 mM α-ketoglutarate and 15 mM NADPH or 17.5 mM NADH in 50 mM Hepes[1] buffer, pH 7.3, at 21 ± 1 °C. At the specified time, the reaction was terminated by acidification to pH 2.0 and ^{15}N spectra taken to observe the formation of [^{15}N]glutamic acid.

(Fig. 5*A*) and glutamate-grown (Fig. 5*C*) cells. The activity in glutamate-grown cells (≤2.7 milliunits·mg^{-1} protein) was repressed by severalfold compared to that in N_2-grown cells (11.4 milliunits·mg^{-1} protein).

DISCUSSION

The results show that, in *B. azotofixans*, the glutamine synthetase/glutamate synthase pathway is the predominant pathway of ammonia assimilation in ammonia-limited cells and a major pathway in ammonia-grown cells. The NADPH-dependent glutamate dehydrogenase activity is detectable at very low levels only in ammonia- and glutamate-grown cells. This suggests that the enzyme has limited physiological function, to participate in assimilating ammonia in the early and midexponential phase of growth when the NH_4^+ concentration in the medium is relatively high (14–22 mM), and in oxidative deamination of glutamate to provide NH_4^+ when glutamate is the sole nitrogen source. Whether *B. azotofixans* is incapable of synthesizing high levels of glutamate dehydrogenase, or does not do so because a high K_m for NH_4^+ limits its utility, cannot be determined at present because its low activity in cell-free extracts precludes measurement of its K_m, and the cells grow poorly when the medium NH_4^+ concentration is very high (60–100 mM). Thus, *B. azotofixans* differs from *B. polymyxa* and *B. macerans*, but resembles other prokaryotes studied previously in the pathway of ammonia assimilation during ammonia limitation. It is significant that N_2-fixing prokaryotes studied to date fall into two groups with respect to the pathway of ammonia assimilation. Thus, they are: (i) *B. polymyxa* and *B. macerans* which have glutamate dehydrogenases with moderate affinity for NH_4^+ and utilize the glutamate dehydrogenase pathway during N_2 fixation (1, 2); and (ii) *B. azotofixans* and other prokaryotes, such as *Clostridium pasteurianum* and *Klebsiella pneumoniae*(4), which utilize the glutamine synthetase/glutamate synthase pathway because they have either barely detectable levels of glutamate dehydrogenase even in ammonia-rich medium, or else glutamate dehydrogenase with an unusually high K_m for NH_4^+ (see Ref. 2). The question raised by our studies on *B. polymyxa* and *B. macerans* (1, 2)—whether, among N_2-fixing prokaryotes that are capable of synthesizing glutamate dehydrogenase with moderate affinity for NH_4^+, the glutamate dehydrogenase pathway is more advantageous than the ATP-requiring glutamine synthetase/glutamate synthase pathway for assimilating ammonia during the energy-demanding process of nitrogen fixation—is an interesting question that requires further investigation among N_2-fixing prokaryotes that possess both pathways.

It is interesting that, whereas the glutamine synthetase activity is derepressed in the ammonia-limited cells, the glutamate synthase activity shows little variation except in glutamate-grown cells where it is repressed. Such apparent lack of derepression of glutamate synthase has been observed in many microorganisms (25). In *B. azotofixans* which has a high intracellular concentration of glutamine (Table I), derepression of glutamate synthase may not be necessary if the enzyme has very high affinities for substrates. Purified glutamate synthases from other *Bacillus* species have K_m values of 0.1–0.18 mM for glutamine, 0.05–0.09 mM for α-ketoglutarate, and 0.007 mM for NADPH (24, 26, 27), whereas purified glutamine synthetases from *Bacillus* species are remarkably similar in having 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 (28–30). Thus, while derepression of glutamine synthetase may be necessary to optimize the assimilation of low concentrations of NH_4^+ into glutamine in nitrate- and N_2-grown cells, the utilization of the glutamine nitrogen for glutamic acid biosynthesis may require only a basal level of glutamate synthase because of the high affinities for substrates and the observed high intracellular concentration of glutamine.

The reliability of the ^{15}N NMR method for distinguishing between the glutamate dehydrogenase and the glutamine synthetase/glutamate synthase pathways is clearly demonstrated by the contrasting kinetic patterns of ^{15}N incorporation into glutamine γ-N and glutamic acid N observed in nitrate-grown *B. azotofixans* (Fig. 1) and *B. polymyxa* (1). The direct *in vivo* method should prove useful for determining the pathway in those microorganisms for which *in vitro* enzyme assays are difficult because of enzyme instability, high background NAD(P)H oxidation, or the heavy slime production observed in many free-living N_2-fixing prokaryotes (31). For organisms in which glutamic acid is formed predominantly by the glutamine synthetase/glutamate synthase pathway, the rate of [^{15}N]glutamic acid biosynthesis observed by NMR represents the *in vivo* glutamate synthase activity. The *in vivo* activity measurement should be particularly useful for ferredoxin-dependent glutamate synthase in algae and plants, whose *in vitro* measurement is quite difficult because it requires the isolation of species-specific ferredoxin, as well as separation of the product glutamate (32).

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[1] The abbreviation used is: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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