Glutamate Biosynthesis in Bacillus azotofixans

15N 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 15N 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 pathway and the glutamate dehydrogenase pathway 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 N2-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 15N NMR for measuring in vivo glutamate synthase activity in the cell.

Our recent studies of the pathways of ammonia assimilation into glutamic acid in N2-fixing Bacillus showed that the glutamate dehydrogenase (GDH) pathway:

\[ \text{NH}_3 + \alpha\text{-ketoglutarate} + \text{NAD(P)}^+ \xrightarrow{\text{GDH}} \text{l-glutamate} + \text{NAD(P)}^+ + \text{H}_2\text{O} \]

is the predominant pathway in N2-fixing cells of Bacillus polymyxa (1) and a major pathway in in those of Bacillus macerans (2). This is in marked contrast to other N2-fixing prokaryotes studied previously which have been shown to assimilate ammonia by the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway:

\[ \text{NH}_3 + \text{l-glutamate} + \text{ATP} \xrightarrow{\text{GS}} \text{l-glutamine} + \text{ADP} + \text{P}_i \]

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 \(\text{NH}_3\) of 2.9 and 2.2 mM, respectively, whereas all other N2-fixing prokaryotes studied previously (4) have either a glutamate dehydrogenase with unusually high \(K_m\) (>11 mM) for \(\text{NH}_3\) 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 \(\text{NH}_3\) 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 \(\text{NH}_3\) fixation, particularly for anaerobic N2 fixers that must generate ATP through the inefficient process of fermentation. By contrast, N2-fixing prokaryotes lacking glutamate dehydrogenase or having a glutamate dehydrogenase with a very high \(K_m\) for \(\text{NH}_3\) must by necessity assimilate ammonia by the glutamine synthetase/glutamate synthase pathway.

Recently, a new N2-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 N2-fixing Bacillus species.

15N nuclear magnetic resonance (NMR) spectroscopy is useful for determining whether 15NH3 is assimilated into glutamic acid by the glutamate dehydrogenase or the glutamine synthetase/glutamate synthase pathway through observation of time-dependent assimilation of 15N into \(\gamma\)-N of glutamine and glutamic acid N in cells incubated with 15N-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 15N NMR in combination with measurements of the specific activities of ammonia-assimilating enzymes.

**EXPERIMENTAL PROCEDURES**

Strains, Media, and Growth—B. azotofixans ATCC 55681, 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 NH₄Cl (22 mM), KNO₃ (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 N₂ fixation, the nitrogen-free medium of Hino and Wilson (5) was used with D-mannitol (60 mM) substituted for sucrose to reduce slime, CaCl₂.2H₂O (66 mg/liter) for CaCO₃, and 0.1 M K₂HPO₄. The extracellular 15N-metabolites were extracted with aqueous 80% H₂SO₄ and then analyzed by 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 KH₂PO₄, K₂HPO₄ buffer, pH 7.8, 5 mM a-ketoglutarate, 80 mM NH₄Cl, 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 [15N]glutamic acid was measured by 15N NMR.

Glutamine-synthetase activity was measured by a modification of the radiochemical method of Frusiner and Milner (12) as described previously (1). The Kₘ value of alanine dehydrogenase for NH₃, which was determined by the method of Lineweaver and Burk (see Ref. 13).

The intracellular NH₃ concentration in N₂-fixing cells was determined on a duplicate culture as described previously (1). For ammonia-grown cells, the intracellular NH₃ concentration could not be determined because the amount of NH₃ trapped in the residual slime in the unwashed cell pellet was large relative to intracellular NH₃.

Chemicals—[15N]NH₄Cl (99% 15N) and K¹⁵NO₃ (98% 15N) were purchased from Cambridge Isotope Laboratories, and [1-¹⁵N]glutamine (95% 15N) 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-

| Nitrogen source | Carbon source | Doubling time (h) | Intracellular concn. of metabolites (mM) | Specific activity (milliunits/mg protein) | In vivo rate (Glu) |
|-----------------|---------------|------------------|-----------------------------------------|------------------------------------------|------------------|
| NH₄⁺            | Mannitol      | 2.8              | 63                                      | 6.8 ± 0.4                                 | 186 ± 68         | 12.5             |
|                 | Glucose       | 2.7              | 63                                      | 18.4                                    |                  |                  |
| NO₃⁻            | Mannitol      | 4.4              | >30                                    | ND                                      |                  |                  |
|                 | Glucose       | 8.1              |                                       | 29.1                                    |                  |                  |
| Glu             | Mannitol      | 7.5              | 11                                      | 4.6                                    |                  |                  |
|                 | Al₂O₃         | 9.1              | 1.2                                     | 3272 ± 472                              |                  |                  |
|                 | Sucrose       | 9.8              | 0.4                                     | 11.4                                    |                  |                  |

*At 21 ± 1 °C.

1 GAH, glutamate dehydrogenase; GS, glutamine synthetase; GOGAT, glutamate synthase; ADH, alanine dehydrogenase.

2 Measured spectrophotometrically.

3 Calculated from the peak intensities in the 30-min spectra in Fig. 1A.

4 ND, not detectable.

5 Measured by 15N NMR.

TABLE I

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

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Ammonia-grown cells or for sucrose in N2-fixing cells (Table I). Thus, its growth is not limited when D-mannitol for D-glucose has no effect on 8519 (990 milliunits mg⁻¹ protein (mannitol)) versus 98 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 NH₃ 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 NH₃ concentration in N₂-fixing cells was found to be 0.4 ± 0.18 mM. Although the intracellular NH₃ 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 were growing under ammonia-limited and ammonia-rich conditions, respectively, as was found for B. polymyxa (1).

### Ammonia Assimilation in Nitrate- and N₂-grown Cells—

**Fig. 1A** shows the biosynthesis of [α-¹⁵N]glutamine and [¹⁵N]glutamic acid in nitrate-grown B. azotofixans as observed by NMR at 3, 8, 18, and 30 min after transfer to NO₃⁻ medium. The ¹⁵N peaks were assigned on the basis of previous work (1, 16). The NO₃⁻ taken up by the cell, is converted by nitrate reductase to NO⁻ which, in turn, is reduced by nitrite reductase to NH₃. After 3 min, as a result of NH₃ assimilation, 54–68% of the glutamine pool was ¹⁵N-labeled in the γ-N (peak at 263.6 ppm) whereas only 6% of the glutamic acid was ¹⁵N-labeled relative to the [¹⁵N]glutamic acid pool observed after 30 min. The observed ±12% variation in the [γ-¹⁵N]glutamine peak intensities between the 18- and 30-min spectra probably arises from experimental error in the extraction of ¹⁵N metabolites. The average of the two peak intensities was taken to represent fully ¹⁵N-labeled glutamine in N₂, 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-¹⁵N]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 ¹⁵N-labeled over a period of 30 min (peak at 335.6 ppm). By 18–30 min, a part of the [¹⁵N]glutamic acid pool had been recycled by the glutamate-synthetase reaction to form [α,γ-¹⁵N]glutamine whose α-¹⁵N peak (334.93 ppm) was resolved from the α-amino ¹⁵N peak of glutamic acid as shown in the expanded-scale spectrum. The extensive ¹⁵N-labeling of the glutamine γ-N prior to that of the glutamic acid N strongly suggests that ¹⁵NH₃ is assimilated mainly by the glutamine synthetase/glutamate synthase pathway in nitrate-grown cells.

**Fig. 1B** shows the effect of inhibitors of glutamine synthetase and glutamate synthase on the biosynthesis of [α-¹⁵N]glutamine and [¹⁵N]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 NO₃⁻ resulted in 95% inhibition of the biosynthesis of [¹⁵N]glutamic acid as indicated by the decrease in its peak intensity compared to that in the control (Fig. 1B). DL-Methionine DL-sulfoximine was added at the high concentration because at 4 mM concentration (in the absence of azaserine), ¹⁵NH₃ 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 [¹⁵N]glutamic acid (in nmol mg⁻¹ protein) calculated from the observed peak intensities in Fig. 1A. The nmols of [¹⁵N]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 [¹⁵N]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 [¹⁵N]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 0.5 nmol [¹⁵N]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/glutamate synthase pathway, the rate of [¹⁵N]glutamic acid biosynthesis, when its substrate glutamine has become fully [¹⁵N]-
that in nitrate-grown cells (Fig. 2). The time-dependent incorporation of \( ^{15} \text{NH}_3 \) into glutamine and glutamic acid in ammonia-grown cells was 29.1 and 15.4 milliunits \( \text{mg}^{-1} \) 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 \textit{B. azotofixans}. The in \textit{vitro} specific activity of glutamate synthase, 15.4 milliunits \( \text{mg}^{-1} \) protein, is reasonably close to the in \textit{vitro} activity, 11.7 milliunits \( \text{mg}^{-1} \) protein observed by NMR. This shows that the enzyme is stable under the assay conditions and that the \textit{in vivo} activity is not substantially limited, compared to the \textit{in vitro} activity, by intracellular concentrations of substrate.

This is understandable because the intracellular concentration of glutamine is 11 mM (Table I) and the glutamine synthetase of \textit{B. azotofixans} is expected to have low \( K_m \) values (<0.2 mM) for its substrates as do the glutamate synthases of other \textit{Bacillus} species (see “Discussion”).

In \textit{N}_2-fixing cells, the glutamate dehydrogenase activity was undetectable whereas glutamine synthetase and glutamate synthase activities were 38 and 11.4 milliunits \( \text{mg}^{-1} \) protein, respectively. The result strongly suggests that in \textit{N}_2-fixing cells of \textit{B. azotofixans}, ammonia is assimilated predominantly by the glutamine synthetase/glutamate synthase pathway.

\section*{Ammonia Assimilation in Ammonia-grown Cells—}
The time-dependent incorporation of \( ^{15} \text{NH}_3 \) into glutamine and glutamic acid in ammonia-grown cells, as observed by NMR at 3, 8, 16, and 24 min after transfer to \( ^{15} \text{NH}_3 \) medium, is shown in Fig. 3. At 3 min, approximately 70% of the glutamine pool was \( ^{15} \text{N} \)-labeled in the \( \gamma \)-N, whereas for glutamic acid, only 18% was \( ^{15} \text{N} \)-labeled relative to the pool at 24 min. The extensive \( ^{15} \text{N} \)-labeling of glutamine \( \gamma \)-N, combined with the slightly faster \( ^{15} \text{N} \)-labeling of glutamic acid compared to that in nitrate-grown cells (Fig. 1A) 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 \( ^{15} \text{N} \)-glutamic acid. The result rules out glutamate dehydrogenase as the predominant pathway because, in such a case, the glutamic acid pool would have been rapidly saturated with \( ^{15} \text{N} \), prior to the glutamine pool, as observed for ammonia-grown \textit{B. polymyxa} (1). The rate of biosynthesis of \( ^{15} \text{N} \)-glutamic acid in ammonia-grown cells was found to be 12.5 ± 0.9 nmol \( \text{min}^{-1} \text{mg}^{-1} \) 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 \( \text{mg}^{-1} \) protein at 60 Klett/ml and 6.8 ± 0.4 milliunits \( \text{mg}^{-1} \) protein at 112 Klett/ml but had no detectable activity at 170 Klett/ml. The \( K_m \) of glutamate dehydrogenase for \( \text{NH}_3 \) could not be determined because of the low activity. The ammonia-grown cells had glutamine synthetase activity of 18.4 milliunits \( \text{mg}^{-1} \) protein and glutamate synthase activity of 16.8 milliunits \( \text{mg}^{-1} \) protein, as well as alanine dehydrogenase activity of 186 ± 68 milliunits \( \text{mg}^{-1} \) 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-glutamate 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 \( ^{15} \text{NH}_3 \) into glutamic acid and alanine was studied in the presence of the following inhibitors: L-methionine DL-sulfoximine (glutamate synthase inhibitor) + azaserine (glutamate synthase inhibitor), aminooxyacetate (alanine-glutamate transaminase inhibitor (20)), and glutarate (an inhibitor of glutamate dehydrogenase (21) and, to a lesser extent, of glutamate synthase). Through \textit{in vitro} assays, glutarate, a structural analog of a-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 \textit{B. polymyxa} (1), and glutamate synthase by 36% in \textit{B. azotofixans}.

Fig. 4 shows \( ^{15} \text{N} \) NMR spectra of ammonia-grown \textit{B. azotofixans} incubated with \( ^{15} \text{NH}_3 \) 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 \( ^{15} \text{N} \)-glutamic acid decreased by 30–40%. The inhibition of \( ^{15} \text{N} \)-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 \( \alpha \)-amino \( ^{15} \text{N} \) 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
Fig. 4. 15N 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 mM glutarate (D), or 4 mM aminooxyacetate (F) before the addition of 15NH3 (16 mM) and incubation for 30 min. Each spectrum in C–F represents an accumulation of 300 scans. Insets to C and D show 15N spectra of the cells in C and D obtained after 3000 scans for comparison of alanine peak intensities.

Alanine dehydrogenase was found to have an apparent K_m of 7.6–18 mM for NH3; a more precise determination was precluded by nonlinearity of the Lineweaver-Burk plot of 1/vo versus 1/[NH3]. The low affinity for NH3 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 glutamate, formation of [15N]aspartic acid (peak at 336.4 ppm in Fig. 4D, inset) increased substantially compared to the control. The cause of this increase is unknown at present, but direct incorporation of 15NH3 into aspartic acid by the aspartate dehydrogenase-catalyzed reaction with oxaloacetate or by the aspartate-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 [15N]aspartic acid due to inhibition of transamination to 15N 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 [15N]glutamic acid from [y-15N]glutamine with NADPH (Fig. 5A) or NADH (Fig. 5B) on addition of cell-free extracts of N2-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* PC1 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 N2-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 [15N] glutamate from [y-15N]glutamine was compared for N2-fixing
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studied previously in the pathway of ammonia assimilation during ammonia limitation. It is significant that N2-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 NH3 and utilize the glutamate dehydrogenase pathway during N2 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 Km for NH3 (see Ref. 2). The question raised by our studies on B. polymyxa and B. macerans (1, 2)—whether, among N2-fixing prokaryotes that are capable of synthesizing glutamate dehydrogenase with moderate affinity for NH3, 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 N2-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 glutamine synthetase 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 Kms 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 Kms values of 0.3–0.4 mM for NH3, 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 NH3 into glutamine in nitrate- and N2-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 15N NMR method for distinguishing between the glutamate dehydrogenase and the glutamine synthetase/glutamate synthase pathways is clearly demonstrated by the contrasting kinetic patterns of 15N 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 N2-fixing prokaryotes (31). For organisms in which glutamic acid is formed predominantly by the glutamine synthetase/glutamate synthase pathway, the rate of 15N glutamic acid biosynthesis observed by NMR represents the in vivo glutamine 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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The abbreviation used is: Hepes, N'-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
REFERENCES

1. Kanamori, K., Weiss, R. L., and Roberts, J. D. (1987) J. Biol. Chem. 262, 11038–11045
2. Kanamori, K., Weiss, R. L., and Roberts, J. D. (1987) J. Bacteriol. 169, 4692–4695
3. Harder, W., and Dijkhuizen, L. (1983) Annu. Rev. Microbiol. 37, 1–23
4. Kleiner, D., Phillips, S., and Fitzke, E. (1981) in Biology of Inorganic Nitrogen and Sulfur (Bothe, H., and Trebst, A., eds.) Springer-Verlag, New York
5. Seldin, L., van Elsas, J. D., and Penido, E. G. C. (1984) Int. J. Syst. Bacteriol. 34, 451–456
6. Hino, S., and Wilson, P. W. (1958) J. Bacteriol. 75, 403–408
7. Kanamori, K., and Roberts, J. D. (1983) Acc. Chem. Res. 16, 35–41
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
9. Meers, J. L., Tempest, D. W., and Brown, C. M. (1970) J. Gen. Microbiol. 64, 187–194
10. Phibbs, P. V., Jr., and Bernlohr, R. W. (1971) J. Bacteriol. 106, 375–385
11. Yoshida, A., and Freese, E. (1970) Methods Enzymol. 17, 176–181
12. Prusiner, S., and Milner, L. (1970) Anal. Biochem. 37, 429–438
13. Dixon, M., and Webb, E. C. (1964) Enzymes, 2nd ed. p. 21, Longmans, London
14. Emerich, D. W., and Burris, R. H. (1978) Biochim. Biophys. Acta 536, 172–183
15. Gordon, R. E., Haynes, W. C., and Pang, C. H. (1973) Agriculture Handbook 427, p. 12, Agriculture Research Service, U.S. Department of Agriculture, Washington, D. C.
16. Legerton, T. L., Kanamori, K., Weiss, R. L., and Roberts, J. D. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 1495–1498
17. Miflin, B. J., and Lea, P. J. (1975) Biochem. J. 149, 403–409
18. Ronzio, R. A., Rowe, W. B., and Meister, A. (1969) Biochemistry 8, 1066–1075
19. Miflin, B. J., and Lea, P. J. (1976) Biochemistry 15, 873–885
20. Hopper, S., and Segal, H. L. (1964) Arch. Biochem. Biophys. 105, 501–505
21. Caughey, W. S., Smiley, J. D., and Hellerman, L. (1957) J. Biol. Chem. 224, 591–607
22. Schmidt, C. N. G., and Jervis, L. (1981) Biochem. Soc. Trans. 9, 454
23. Tanaka, N., and Hanson, R. S. (1975) J. Bacteriol. 122, 215–223
24. Matsuoka, K., and Kimura, K. (1986) J. Biochem. 99, 1087–1100
25. Tyler, B. (1978) Annu. Rev. Biochem. 47, 1127–1162
26. Bernlohr, R. W., Schreier, H. J., and Donohue, T. J. (1984) Curr. Top. Cell. Regul. 24, 145–152
27. Hemmila, I. A., and Mantsala, P. I. (1978) Biochem. J. 173, 45–52
28. Deuel, T. F., and Stadtman, E. R. (1970) J. Biol. Chem. 245, 5206–5213
29. Donohue, T. J., and Bernlohr, R. W. (1981) J. Bacteriol. 147, 589–601
30. Matsuoka, K., Kurebayashi, T., and Kimura, K. (1985) J. Biochem. (Tokyo) 98, 1211–1219
31. Mulder, E. G. (1975) in Nitrogen Fixation by Free-living Microorganisms (Stewart, W. D. P., ed) pp. 3–28, Cambridge University Press, London
32. Suzuki, A., and Gadal, P. (1982) Plant Physiol. 69, 848–852