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Role of Glutamate Dehydrogenase in Ammonia Assimilation in Nitrogen-Fixing *Bacillus macerans*†

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Pathways of ammonia assimilation into glutamic acid in *Bacillus macerans* were investigated by measurements of the specific activities of glutamate dehydrogenase (GDH), glutamine synthetase, and glutamate synthase. In ammonia-rich medium, GDH was the predominant pathway of ammonia assimilation. In nitrogen-fixing cells in which the intracellular NH_4^+ concentration was 1.4 ± 0.5 mM, the activity of GDH with a K_m of 2.2 mM for NH_4^+ was found to be severalfold higher than that of glutamate synthase. The result suggests that GDH plays a significant role in the assimilation of NH_4^+ in N_2-fixing *B. macerans*.

Two major pathways of ammonia assimilation into glutamic acid are the glutamate dehydrogenase (GDH) pathway

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

and the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway

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

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

Most microorganisms utilize the GDH pathway in ammonia-rich medium. In ammonia-limited medium, such as during growth in low concentrations of ammonia or use of nitrate or molecular nitrogen as a nitrogen source, most microorganisms derepress GS, which has a much lower K_m for NH_4^+ than GDH, and assimilate NH_4^+ by the GS-GOGAT pathway. All N_2-fixing procaryotes reported to date have been shown to assimilate ammonia by the GS-GOGAT pathway during N_2 fixation (4, 17, 22).

However, our recent study of the pathways of ammonia assimilation in nitrogen-fixing *Bacillus polymyxa* showed that the activity of GDH was 20-fold higher than that of GS and GOGAT in cell extracts of N_2-fixing *B. polymyxa* (14). This strongly suggested that *B. polymyxa*, unlike all N_2-fixing procaryotes reported previously, assimilates ammonia predominantly by the GDH pathway during N_2 fixation. To investigate whether this is characteristic only of *B. polymyxa* or is also true of other N_2-fixing species of *Bacillus*, we have undertaken a study of another N_2-fixing species, *Bacillus macerans*. We report here a study of the pathways of ammonia assimilation in ammonia-grown and N_2-fixing *B. macerans*.

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MATERIALS AND METHODS

Strains, media, and growth. A wild-type strain of *B. macerans*, ATCC 8515, was obtained from the American Type Culture Collection.

For growth on ammonia (22 mM) as the nitrogen source, the growth medium previously described (14) was supplemented with thiamine hydrochloride (100 μg/liter). Growth was monitored in a Klett-Summerson colorimeter with a no. 540 filter. Batch cultures (250 ml) were grown aerobically in baffled flasks on a shaker at 30°C from an inoculum of 2 to 3 Klett units to the mid-exponential phase.

For N_2 fixation, the nitrogen-free medium previously described (14) was supplemented with thiamine hydrochloride (100 μg/liter) and yeast extract (40 mg/liter) (29). The cultures were grown anaerobically at 30°C from a 2.5% inoculum to the mid-exponential phase under a continuous flow (200 ml/min) of high-purity (99.99%) N_2.

Intracellular NH_4^+ concentrations in ammonia-grown and N_2-fixing cells were determined on duplicate cultures as described previously (14).

Enzyme assays. The cell extracts for enzyme assays were prepared by harvesting the cells at mid-exponential phase (168 ± 10 Klett units/ml for NH_4^+-grown and 145 ± 25 Klett units/ml for N_2-fixing cells), washing with the specified buffers, and disrupting the cells by sonication as described previously (14). All enzyme assays were performed at 20°C within 1 h of harvesting the cells and were performed on duplicate cultures. Protein was measured by the method of Lowry et al. (18), with bovine serum albumin as the standard.

GDH activity was determined spectrophotometrically by measuring the rate of oxidation of NADPH by a modification of the standard procedure (20). The reaction mixture contained 50 mM Tris hydrochloride (pH 7.8), 5 mM 2-mercaptoethanol, 0.1 mM EDTA, 5 mM α-ketoglutarate, 80 mM NH_4Cl, and 0.3 mM NADPH. Low activities of NADPH oxidase present in crude extracts were determined with appropriate reagent blanks and were subtracted. Specific activities are reported as milliunits, i.e., nanomoles of NADPH oxidized per minute, per milligram of protein. K_ms

TABLE 1. Ammonia-assimilating enzymes in *B. macerans*

Nitrogen source	Doubling time (h)	Intracellular NH_4^+ concn (mM)	Sp act (mU/mg of protein)		
			GDH	GS	GOGAT
Ammonia	3.2	10.1	276 ± 34	40	0.8
N_2	23	1.4 ± 0.5	21 ± 5	16	4.5

of GDH for ammonia and α-ketoglutarate were determined by the method of Lineweaver and Burk.

GS activity was measured by a modification of the radiochemical method of Prusiner and Milner (25) as described previously (14).

GOGAT activity was assayed by measuring the rate of formation of [^{15}N]glutamate from [γ-^{15}N]glutamine by ^{15}N nuclear magnetic resonance (NMR) spectroscopy. The reaction was initiated by addition of cell extract to a 2-ml assay solution containing 5 mM [γ-^{15}N]glutamine, 5 mM α-ketoglutarate, and 15 mM NADPH in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.3) at 20°C. The reaction was terminated at appropriate time intervals by acidification to pH 2.5. After removal of denatured protein by centrifugation, addition of 10 mg of EDTA, and neutralization to pH 7, ^{15}N NMR spectra were taken. Activity is reported as milliunits (nanomoles of [^{15}N]glutamate formed per minute) per milligram of protein.

NMR experiments. 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 per million upfield from 1 M $H^{15}NO_3$. The operating conditions were a 20.5-μs (70° flip angle) pulse width and proton decoupling by WALTZ-16 composite pulse sequence.

Chemicals. L-[γ-^{15}N]glutamine (95% ^{15}N) was purchased from MSD Isotopes, and L-[^{15}N]glutamic acid (99% ^{15}N) was from Cambridge Isotopes. All other chemicals were reagent grade.

RESULTS

The wild-type strain of *B. macerans* (ATCC 8515) grew aerobically in a batch culture having an initial NH_4^+ concentration of 22 mM with a doubling time of 3.2 h and fixed N_2 anaerobically with a doubling time of 23 h at 30°C. N_2 is reduced to NH_4^+ by the nitrogenase complex. The intracellular NH_4^+ concentration of N_2-fixing *B. macerans* was 1.4 ± 0.5 mM, which was significantly lower than the 10.1 mM observed in ammonia-grown cells at mid-exponential phase (Table 1).

GDH of *B. macerans*, catalyzing the biosynthetic reaction, was found to be specific for NADPH; no NADH-dependent GDH activity could be detected. K_ms of GDH were found to be 2.2 mM for NH_4^+ and 0.38 mM for α-ketoglutarate. At enzyme-saturating concentrations of NH_4^+, GDH activity was found to be optimal when the concentration of α-ketoglutarate was 5 mM; at higher concentrations, α-ketoglutarate had an inhibitory effect on the activity of GDH. Similar inhibition of GDH by α-ketoglutarate at concentrations 10 times higher than the K_m has been reported for *Bacillus licheniformis* (24). Therefore, specific activities of GDH were measured with an α-ketoglutarate concentration of 5 mM.

GOGAT activity in crude cell extracts was too low to be detected through measurement of the rate of oxidation of NADPH. In a standard assay solution containing 5 mM L-glutamine, 5 mM α-ketoglutarate, and 0.3 mM NADPH in 50 mM HEPES buffer (pH 7.3), oxidation of NADPH by NADPH oxidase and other enzymes in the extract was too rapid relative to that by GOGAT for quantitative determination. Dialysis of the cell extract against two changes (500 ml each) of 0.1 M Tris hydrochloride buffer (pH 8.0) supplemented with 10 mM 2-mercaptoethanol at 4°C reduced the background oxidation of NADPH, but the GOGAT activity was still too low for accurate measurement. Activity was not increased by increasing the pH of the assay solution to 7.8. No activity was detected with NADH as the coenzyme.

The GOGAT activity was therefore measured by another method which allows addition of excess NADPH and detection of the product without separation from the substrates. The rate of formation of [^{15}N]glutamate from [γ-^{15}N]glutamine was measured in an assay solution containing 5 mM [γ-^{15}N]glutamine, 5 mM α-ketoglutarate, and 15 mM NADPH in 50 mM HEPES buffer at pH 7.3. Figure 1 shows the ^{15}N NMR spectra of [^{15}N]glutamate formed in reaction mixtures at 15, 30, and 100 min (Fig. 1A, B, and C) after the addition of the cell extracts of N_2-fixing *B. macerans*. The ^{15}N peaks were assigned on the basis of our previous work (14). The formation of [^{15}N]glutamate, as observed from the

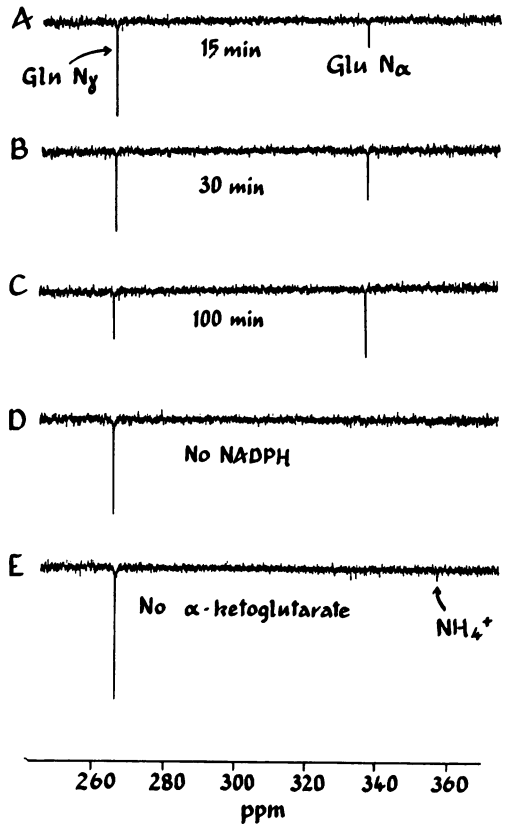

FIG. 1. ^{15}N NMR spectra of GOGAT assay solutions containing 5 mM [γ-^{15}N]glutamine, 5 mM α-ketoglutarate, and 15 mM NADPH at various time intervals after addition of cell extracts of N_2-fixing *B. macerans*. (A, B, and C) Complete assay solutions at 15, 30, and 100 min, respectively. (D) Assay solution without NADPH at 100 min. (E) Assay solution without α-ketoglutarate at 100 min. A to D were taken with a delay of 7 s to observe the formation of [^{15}N]glutamate, E with a delay of 40 s to detect $^{15}NH_4^+$ as well.

increase in the intensity of its peak at 335.1 ppm, was linear with time for 30 min. No [^{15}N]glutamate was formed when NADPH or α-ketoglutarate was omitted from the assay solution (Fig. 1D and E).

The amount of [^{15}N]glutamate formed in each assay solution was determined from the observed peak intensity as follows. [γ-^{15}N]glutamine and [^{15}N]glutamate were found to recover 83 and 82% of the equilibrium peak intensity, respectively, under the operating conditions (a relaxation delay of 7 s), and each had a nuclear Overhauser enhancement value of −3.9. Thus, equimolar quantities of [γ-^{15}N]glutamine and [^{15}N]glutamate have essentially the same ^{15}N peak intensities. From the observed relative peak intensities of [γ-^{15}N]glutamine and [^{15}N]glutamate and the initial quantity (10 μmol) of [γ-^{15}N]glutamine present in the assay solution, the amount of [^{15}N]glutamate formed in each assay solution was calculated. GOGAT activity is expressed as nanomoles of [^{15}N]glutamate formed per minute per milligram of protein.

To investigate whether enzymatic or chemical hydrolysis of [γ-^{15}N]glutamine to $^{15}NH_4^+$ and unlabeled glutamate had occurred during incubation with the cell extracts, a ^{15}N spectrum of the reaction mixture without α-ketoglutarate was taken with a relaxation delay of 40 s to permit detection of the slowly relaxing ^{15}N nucleus of $^{15}NH_4^+$. This delay was shown in a separate experiment to allow 86% recovery of the equilibrium peak intensity for $^{15}NH_4^+$, and its ^{15}N nucleus was found to have a nuclear Overhauser enhancement value of −3.0. A barely detectable $^{15}NH_4^+$ peak at 353 ppm was found (Fig. 1E). This result indicates that hydrolysis of [γ-^{15}N]glutamine during the assay was negligible. Therefore, [^{15}N]glutamate observed in the complete reaction mixtures (Fig. 1A to C) was formed predominantly by the GOGAT-catalyzed reaction of [γ-^{15}N]glutamine with α-ketoglutarate and not by a GDH-catalyzed reaction of α-ketoglutarate with $^{15}NH_4^+$ released by hydrolysis of [γ-^{15}N]glutamine. The residual NADPH concentration in the complete reaction mixture at 100 min, determined by UV absorbance at 340 nm, was 2.6 mM. Therefore, the observed GOGAT activity was not limited by oxidation of NADPH by other enzymes in the cell extracts.

The specific activities of GDH, GS, and GOGAT in ammonia-grown and N_2-fixing *B. macerans* are listed in Table 1. In ammonia-grown cells, the GDH activity, at 276 ± 34 mU/mg of protein, was approximately 345-fold higher than the GOGAT activity. Therefore, GDH is the predominant pathway of ammonia assimilation.

In N_2-fixing cells, the GDH activity, at 21 ± 5 mU/mg of protein, was four- to fivefold higher than the GOGAT activity, 4.5 mU/mg of protein. While extrapolation from in vitro results to the in vivo situation must be performed with caution (7), it is possible to make an approximate estimate of the contribution of the GDH pathway to glutamate synthesis in the cell by taking into account the K_m of GDH for NH_4^+ and the intracellular NH_4^+ concentration. At an NH_4^+ concentration of 1.4 ± 0.5 mM, which prevails in N_2-fixing cells (Table 1), the rate of assimilation of NH_4^+ by GDH with the K_m of 2.2 mM for NH_4^+ is expected to be 38 ± 9% of the optimal rate observed in the in vitro assay, on the basis of the Michaelis-Menten equation (21 × 0.38 = 8 nmol/min per mg of protein). Assuming that the GOGAT of *B. macerans*, like those of the other *Bacillus* species (2, 13), has very low K_ms for its substrates and can therefore operate at the rate observed in the in vitro assay, 4.5 nmol/min per mg of protein, the contribution of the GDH pathway to the total glutamate biosynthesis is approximately 60%. This suggests that the GDH pathway plays a major, though not a predominant, role in the ammonia assimilation in N_2-fixing cells.

DISCUSSION

The results suggest that GDH plays a substantial role in ammonia assimilation during N_2 fixation in *B. macerans* and a predominant role in *B. polymyxa* (14). In both species, the GOGAT level is low in both ammonia-grown and N_2-fixing cells. This behavior of N_2-fixing *Bacillus* species differs from that of other free-living N_2-fixing procaryotes studied to date, in which the GOGAT activity was found to be significantly higher (22) than, or at least comparable (6, 9) to, that of GDH during N_2 fixation.

It is important that the N_2-fixing procaryotes that have been studied previously and reported to assimilate NH_4^+ by the GS-GOGAT pathway either have undetectable levels of GDH even in ammonia-rich medium (*Clostridium pasteurianum* [4, 21] and *Azotobacter vinelandii* [15]) or have GDH with very high K_ms for NH_4^+ (*Klebsiella pneumoniae*, 12 mM [16]; *Rhodospirillum rubrum*, 11.5 mM [6]; and *Chromatium* sp. strain D, 6 to 16 mM [5]). Such enzymes are clearly incapable of assimilating low concentrations of NH_4^+. *B. macerans* and *B. polymyxa* have GDHs with K_ms for NH_4^+ of 2.2 and 2.9 mM, respectively, which permits slow assimilation of NH_4^+, whose intracellular concentration is 1.4 ± 0.5 mM in N_2-fixing cells. Thus, GDH may play a significant role in ammonia assimilation in organisms that have a GDH with a K_m for NH_4^+ in the common range of 1 to 5 mM (12, 27), but have very low levels of GOGAT. Among N_2-fixing procaryotes, such species have not been reported, to the best of our knowledge, except for the *Bacillus* species studied here. However, a biosynthetic role for GDH has been reported for *Escherichia coli* (26), yeasts (4); and *Bacteroides fragilis* (30) grown on low concentrations of NH_4^+.

The GDH activities of both ammonia-grown and N_2-fixing *B. macerans* (Table 1) are considerably lower than those of corresponding cultures of *B. polymyxa* (798 and 313 mU/mg of protein, respectively). *B. macerans*, because its GDH has a much lower K_m for α-ketoglutarate (0.38 mM) than that of *B. polymyxa* (1.4 mM), may well be more efficient in glutamate synthesis and thus may require lower levels of GDH for growth. The fact that the doubling times of *B. macerans* (Table 1) are very close to those of *B. polymyxa* (3.2 h for ammonia-grown and 25 h for N_2-fixing cells) under the same growth conditions is consistent with this interpretation.

It remains to be determined whether *B. macerans* and *B. polymyxa* are incapable of synthesizing high levels of GOGAT under any growth condition or utilize the GDH pathway during ammonia limitation because of some advantage to the cell. The mechanism of regulation of ammonia-assimilating enzymes in the gram-positive *Bacillus* species appears to differ from the complex mechanisms of regulation observed in the gram-negative enteric bacteria (19, 27). The question has attracted interest, but relatively little is known at present. The levels of ammonia-assimilating enzymes have been shown to vary with the nitrogen source in *B. licheniformis* (2) and *Bacillus subtilis* (23), and the regulation has been shown to be at the transcriptional level in *B. subtilis* (3, 10). In *B. licheniformis*, pool sizes of metabolites on the assimilatory pathway, NH_4^+, α-ketoglutarate, glutamate, and glutamine, have been shown to be unrelated to the levels of GDH, GS, and GOGAT; therefore, these metabolites, either singly or in combination, do not appear to play a direct

or exclusive role in the synthesis of the enzymes (2). Further physiological and genetic studies are needed to understand the mechanism of regulation of GDH, GS, and GOGAT in the *Bacillus* species.

Although the high affinities of GS and GOGAT for their respective substrates constitute a clear advantage of the pathway for assimilating low concentrations of ammonia, the coupled pathway consumes 1 molecule each of ATP and NAD(P)H to synthesize 1 molecule of glutamate from NH_4^+ and α-ketoglutarate, while the GDH pathway utilizes 1 molecule of NADPH to achieve the identical result. The lower energy requirement of the GDH pathway may be advantageous to N_2-fixing cells that require from 4 to 29 ATP molecules (depending on the organism) to reduce 1 molecule of N_2 (1). This can be especially true for *B. polymyxa* and *B. macerans*, which fix N_2 only under strictly anaerobic conditions and therefore must generate ATP through the low-energy-yielding fermentation of glucose to ethanol and acetate (*B. macerans*) or 2, 3-butanediol (*B. polymyxa*) as major products and H_2, CO_2, and acetone as minor products (11, 28). For such organisms, ammonia assimilation by GDH, if it has moderate affinity for NH_4^+, may well be advantageous during N_2 fixation.

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