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Ammonia Assimilation Pathways in Nitrogen-Fixing *Clostridium kluyverii* and *Clostridium butyricum*†

KEIKO KANAMORI,[1,2]* RICHARD L. WEISS,[1] AND JOHN D. ROBERTS[2]

Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90024-1569,[1] and Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California 91125[2]

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Pathways of ammonia assimilation into glutamic acid were investigated in ammonia-grown and N_2-fixing *Clostridium kluyverii* and *Clostridium butyricum* by measuring the specific activities of glutamate dehydrogenase, glutamine synthetase, and glutamate synthase. *C. kluyverii* had NADPH-glutamate dehydrogenase with a K_m of 12.0 mM for NH_4^+. The glutamate dehydrogenase pathway played an important role in ammonia assimilation in ammonia-grown cells but was found to play a minor role relative to that of the glutamine synthetase/NADPH-glutamate synthase pathway in nitrogen-fixing cells when the intracellular NH_4^+ concentration and the low affinity of the enzyme for NH_4^+ were taken into account. In *C. butyricum* grown on glucose-salt medium with ammonia or N_2 as the nitrogen source, glutamate dehydrogenase activity was undetectable, and the glutamine synthetase/NADH-glutamate synthase pathway was the predominant pathway of ammonia assimilation. Under these growth conditions, *C. butyricum* also lacked the activity of glucose-6-phosphate dehydrogenase, which catalyzes the regeneration of NADPH from $NADP^+$. However, high activities of glucose-6-phosphate dehydrogenase as well as of NADPH-glutamate dehydrogenase with a K_m of 2.8 mM for NH_4^+ were present in *C. butyricum* after growth on complex nitrogen and carbon sources. The ammonia-assimilating pathway of N_2-fixing *C. butyricum*, which differs from that of the previously studied *Bacillus polymyxa* and *Bacillus macerans*, is discussed in relation to possible effects of the availability of ATP and of NADPH on ammonia-assimilating pathways.

The N_2-fixing procaryotes studied to date fall into two groups with respect to the pathways of ammonia assimilation; (i) *Bacillus polymyxa* and *Bacillus macerans*, which have glutamate dehydrogenases (GDHs) with K_ms for ammonia of 2.2 to 2.9 mM and utilize the GDH pathway during N_2 fixation (13, 14), and (ii) *Bacillus azotofixans* (15) and other procaryotes such as *Clostridium pasteurianum* (3, 20, 21) and *Klebsiella pneumoniae* (16, 21), which utilize the glutamine synthetase (GS)-glutamate synthase (GOGAT) pathway because they either have barely detectable levels of GDH even in ammonia-rich medium (3, 20) or else GDH with a high K_m for NH_4^+ (16). Our results with *Bacillus* spp. suggest that, for N_2-fixing procaryotes capable of synthesizing GDH with a moderate affinity for NH_4^+, the GDH pathway may be more advantageous than the ATP-requiring GS-GOGAT pathway for assimilating ammonia during the energy-demanding process of nitrogen fixation (14). To determine whether the GDH pathway plays an important role in ammonia assimilation during N_2 fixation in genera other than *Bacillus*, we studied the pathways of ammonia assimilation in two nitrogen-fixing species of obligate anaerobes, *Clostridium kluyverii* and *Clostridium butyricum* (25). *C. kluyverii*, which utilizes ethanol, acetate, and bicarbonate as carbon sources, has GDH activity (27) when grown with NH_4^+ as the nitrogen source, although its K_m for NH_4^+ has not been measured and its coenzyme specificity has been controversial (10, 27). *C. butyricum* grown on complex medium (11) possesses GDH, and measurements of the NADH- and NADPH-dependent GDH activities in crude extracts as a function of NH_4^+ concentration suggest that the enzyme(s) have moderate affinity for NH_4^+ (2).

Studies of NAD(P)H-dependent enzymes such as GDH and GOGAT in the *Clostridium* species have been hampered by the presence in cell extracts of NADH oxidase and NAD(P)H-ferredoxin reductases, which cause very rapid oxidation of NAD(P)H. As a result, measurements of the specific activities and K_ms for substrates of GDH and GOGAT through the standard spectrophotometric method of observing the rate of oxidation of NAD(P)H are very difficult. Assays under anaerobic conditions (8) and/or through detection of [^{14}C]glutamate formed from ^{14}C-labeled substrates (27) have been reported. However, ^{15}N nuclear magnetic resonance (NMR) spectroscopy provides an especially convenient method of measuring the specific activities of these enzymes through observation of the time-dependent formation of the product [^{15}N]glutamic acid from $^{15}NH_4^+$ (GDH) or from [γ-^{15}N]glutamine (GOGAT) without separation from the substrates in assay solutions containing excess NAD(P)H or an NADH-regenerating system. We report here an investigation of the pathways of ammonia assimilation in ammonia-grown and N_2-fixing *C. kluyverii* and *C. butyricum* through measurements of the specific activities of GDH, GS, and GOGAT.

MATERIALS AND METHODS

Strains, media, and growth. *C. kluyverii* (ATCC 8527) and *C. butyricum* (ATCC 8260) were obtained from the American Type Culture Collection. For *C. kluyverii*, the medium (for large-scale culture) described by Stadtman and Burton (26) was used with 22 mM NH_4Cl as the nitrogen source. Growth was monitored in a Klett-Summerson colorimeter with a no. 540 filter. Cells were grown from an inoculum of 2 to 3 Klett units to the midexponential phase (120 Klett units) in a closed vessel at 30°C. For N_2 fixation, the same medium without NH_4Cl was supplemented with $FeSO_4 \cdot 7H_2O$ (85 mg/liter) and $Na_2MoO_4 \cdot 2H_2O$ (30 mg/liter). A

* Corresponding author.
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10% inoculum of culture grown in limited (5 mM) NH_4Cl was added to the N-free medium and grown from 10 Klett units to 60 Klett units at 37°C with a continuous flow (200 ml/min) of N_2 containing 3% CO_2. A continuous supply of CO_2 was found to be essential for growth under this condition. When an uninoculated medium was sparged with N_2 alone, the concentration of HCO_3^-, an essential carbon source for *C. kluyverii* supplied at 20 mM in the medium, was found to decrease to one-half within 3 h (as measured by decrease in the radioactivity of $H^{14}CO_3^-$ added at 0.1 μCi/ml of medium) as the result of CO_2 loss by the reaction $HCO_3^- + H^+ \rightleftharpoons H_2O + CO_2\uparrow$.

C. butyricum was maintained in reinforced *Clostridium* medium (Difco Laboratories) containing (per liter) tryptose (10 g), beef extract (10 g), yeast extract (3 g), dextrose (5 g), NaCl (5 g), starch (1 g), cysteine hydrochloride (0.5 g), sodium acetate (3 g), and Bacto-Agar (Difco) (0.5 g). For growth on NH_4Cl (22 mM) as the nitrogen source, the medium described by Carnahan and Castle (6) was used with the following modifications; $CaCl_2 \cdot H_2O$ (133 mg/liter) was substituted for $CaCO_3$, glucose (20 g/liter) was substituted for sucrose, and K_2HPO_4-KH_2PO_4 buffer was used at 0.1 M. For N_2 fixation, the nitrogen-free medium was supplemented with $FeSO_4 \cdot 7H_2O$ (85 mg/liter) and $Na_2MoO_4 \cdot 2H_2O$ (20 mg/liter). Cultures were grown anaerobically with a continuous flow of N_2 at 37°C from a 1.5% inoculum of ammonia-grown cells to 195 Klett units for large-scale growth on NH_4^+ and from a 6% inoculum of N_2-fixing cells to 107 Klett units for large-scale growth on N_2. The purity of the culture was checked periodically by microscopic observation of smears stained with safranin.

Enzyme assays. The cells were harvested by centrifugation at 14,680 × *g* and washed in 0.1 M Tris hydrochloride buffer (pH 7.4) containing 1 mM cysteine (*C. kluyverii*) or in 50 mM KH_2PO_4 buffer (pH 7.0) containing 0.004% sodium thioglycollate (*C. butyricum*). The cell pellet was either used immediately or stored at −20°C until use. For preparation of cell extracts for GDH and GOGAT assays, the cell pellet was suspended in the same buffer (supplemented with 20 mM mercaptoethanol for *C. kluyverii*) and disrupted by sonication at 4°C as described previously (13). For GS and glucose-6-phosphate dehydrogenase (G6PD) assays, the buffers used for preparation of cell extracts were 0.2 M KH_2PO_4 (pH 7.5) and 5 mM Tris hydrochloride (pH 7.6) containing 10 mM $MgCl_2$, respectively. The cell debris was removed by centrifugation at 36,600 × *g*, and the supernatant containing 5 to 20 mg of protein was used for enzyme assays. Protein was measured by the method of Lowry et al. (19) with bovine serum albumin as the standard. All enzyme assays were performed at 20°C and completed within 1 or 2 h after preparation of the cell extracts.

GS activity was measured by a modification of the radiochemical method of Prusiner and Milner (24) as described previously (13). The assay solution, modified on the basis of the known properties of the GS of *C. pasteurianum* (18), contained the following in a final volume of 1.1 ml: 50 mM 3-(*N*-morpholino)propanesulfonic acid buffer (pH 7.2), 12 mM NH_4Cl, 25 mM glutamic acid with L-[U-^{14}C]glutamic acid (1.72 μCi/ml), 12 mM ATP, 24 mM $MgCl_2$, and 0.1 mM aminooxyacetate.

GOGAT activity was assayed by ^{15}N NMR as described previously (14) with the following modifications. The assay solution contained 5 mM [γ-^{15}N]glutamine, 5 mM α-ketoglutarate, and 7.5 mM NAD(P)H for *C. butyricum* and 15 mM NAD(P)H for *C. kluyverii*. The reaction was performed under N_2 to minimize oxidation of NADH by endogenous NADH oxidase in the cell extracts. For assay of NADPH-GDH activity, cell extracts were added to an assay solution containing 25 mM $^{15}NH_4Cl$, 5 mM α-ketoglutarate, and 15 mM NADPH in 0.1 M Tris buffer (pH 7.0) and incubated under N_2. At various time intervals, a 2-ml sample of the reaction mixture was withdrawn anaerobically, and the reaction was terminated by acidification to pH 2.0. After removal of denatured protein by centrifugation and neutralization to pH 6 to 7, the extent of formation of [^{15}N]glutamic acid was measured by ^{15}N NMR. The amount of [^{15}N]glutamic acid formed in the reaction mixture was calculated from its peak intensity in the NMR spectra by comparison with the peak intensity of a known amount of [^{15}N]glutamic acid. For assay of NADH-GDH activity, the assay solution contained 7.5 mM NADH or an NADH-regenerating system consisting of 20 mM ethanol, 0.27 mM NAD^+, and alcohol dehydrogenase (2.8 U/ml) in Tris buffer at pH 7.8. The system generated NADH to a maximum concentration of approximately 0.15 mM as observed by UV absorbance in the assay solution before the addition of cell extracts. GDH and GOGAT activities are reported as milliunits (nanomoles of [^{15}N]glutamic acid formed per minute) per milligram of protein.

The ^{15}N NMR spectra were obtained at 50.68 MHz as described previously (13). ^{15}N chemical shifts are reported in parts per million upfield from 1 M $H^{15}NO_3$.

When the spectrophotometric method was used to assay for GDH (for *C. butyricum* grown on complex medium), the assay solution described above was modified by decreasing the NAD(P)H concentration to 0.25 mM. The activity for reductive amination is reported as milliunits (nanomoles of NAD[P]H oxidized per min) per milligram of protein. For measurement of the K_m for glutamate of NADPH-GDH in the oxidative deamination reaction, the assay solution contained 0.3 mM $NADP^+$ and L-glutamate at concentrations ranging over 5 to 50 mM in 0.1 M Tris buffer (pH 7.8). The rates of oxidation of NAD(P)H in the absence of substrates for GDH and GOGAT—the "background" oxidation—by cell extracts of *C. kluyverii* and *C. butyricum* were measured in 0.1 M Tris hydrochloride buffer (pH 7.0) containing 0.25 mM NAD(P)H. G6PD activity was measured spectrophotometrically by the standard procedure (29), except that the concentration of $NADP^+$ in the assay solution was 0.3 mM. Activity is reported as milliunits (nanomoles of $NADP^+$ reduced per minute) per milligram of protein.

Intracellular NH_4^+ concentrations in *C. kluyverii* were determined by assaying for NH_4^+ after perchlorate extraction and, for ammonia-grown cells, correcting for the amount of NH_4^+ in the medium trapped in the cell pellet, as described previously (13).

RESULTS

C. kluyverii. Cell extracts of ammonia-grown *C. kluyverii* oxidized NADPH and NADH at the rates of 1,246 ± 323 and 3,000 ± 138 mU/mg of protein, respectively. This high background oxidation precluded spectrophotometric measurements of the GDH and GOGAT activities. The GDH activity was therefore measured through observation of the time-dependent formation of [^{15}N]glutamic acid as described in Materials and Methods. Figure 1 shows representative ^{15}N NMR spectra of the reaction mixtures for ammonia-grown cells (Fig. 1A through D) and N_2-fixing cells (Fig. 1E through G). Significant formation of [^{15}N]glutamic acid (335.1 ppm) was observed in the presence of NADPH (Fig. 1A, B, E, and F) but not in the presence of 15 mM NADH

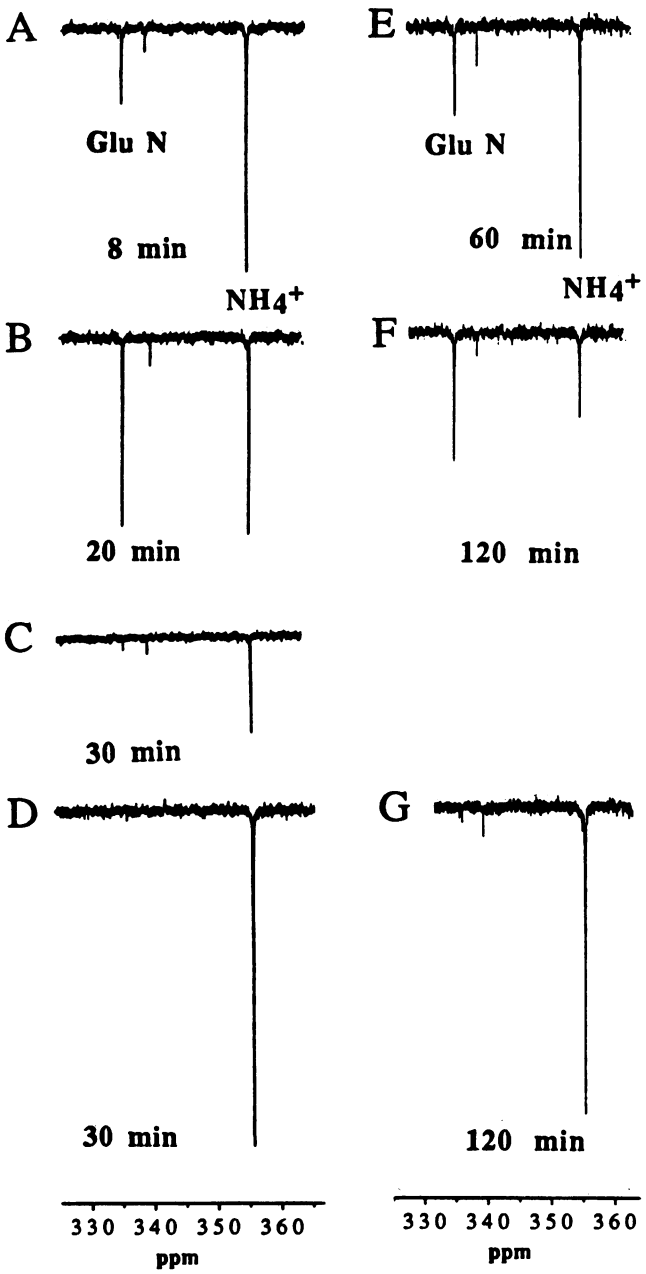

FIG. 1. ^{15}N NMR spectra of GDH assay solutions at various time intervals after addition of cell extracts of ammonia-grown (A through D) and of N_2-fixing (E through G) *C. kluyverii*. Assay preparations contained 15 mM NADPH (A, B, E, F), 15 mM NADH (C), or no coenzyme (D, G). The small peak at 338 ppm represents the nitrogen in the Tris buffer.

(Fig. 1C) or in the absence of coenzyme (Fig. 1D and G). The GDH activity was also assayed in the presence of an NADH-regenerating system that regenerates NADH to a maximum concentration of 0.15 mM, because the activities of NADH-GDHs from many organisms have been shown to be inhibited at high NADH concentrations (9, 31). No [^{15}N]glutamic acid formation was observed (data not shown). The results show that the GDH of *C. kluyverii* is NADPH specific. Figure 2 shows the time-dependent formation of [^{15}N]glutamic acid calculated from the observed peak intensities in the NMR spectra to determine the specific

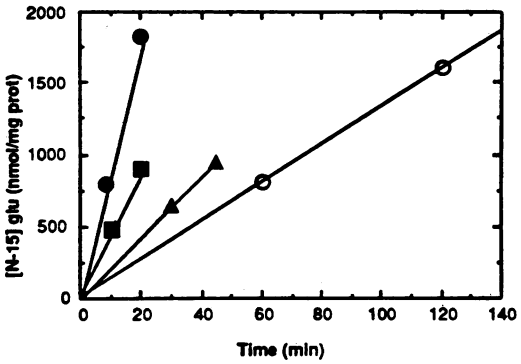

FIG. 2. Time-dependent formation of [^{15}N]glutamic acid after the addition of cell extracts of ammonia-grown *C. kluyverii* to GDH assay solutions with initial $^{15}NH_4^+$ concentrations of 25 mM (●), 5 mM (■), and 2 mM (▲) and after addition of cell extracts of N_2-fixing cells to an assay solution with a $^{15}NH_4^+$ concentration of 25 mM (○).

activity of NADPH-GDH. The activity for NH_4^+-grown *C. kluyverii* was 91 mU (nmol of [^{15}N]glutamic acid formed per min) per mg of protein when assayed with a $^{15}NH_4^+$ concentration of 25 mM. To estimate the affinity of the enzyme for NH_4^+, the activity was measured at various concentrations of NH_4^+. At $^{15}NH_4^+$ concentrations of 5 and 2 mM, the activity decreased to 47 and 20.5 mU/mg of protein, respectively (Fig. 2). An assay with a duplicate culture confirmed that the activity at the NH_4^+ concentration of 5 mM is very close to one-half of that at 25 mM. Lineweaver-Burk plots of $1/[NH_4^+]$ versus $1/v_0$ gave a K_m^{app} of 12.0 mM for NH_4^+. For N_2-fixing *C. kluyverii*, the specific activity of NADPH-GDH, calculated from the data in Fig. 2, was 13.4 mU/mg of protein at a $^{15}NH_4^+$ concentration of 25 mM.

In GOGAT assays performed with NAD(P)H as the coenzyme, [^{15}N]glutamic acid was formed only in the presence of NADPH. NADPH-GOGAT activities were 32 and 5.4 mU/mg of protein for ammonia-grown and N_2-fixing *C. kluyverii*, respectively.

Table 1 lists the specific activities of GDH, GS, and GOGAT and the average intracellular NH_4^+ concentrations in ammonia-grown and N_2-fixing *C. kluyverii*. To estimate the actual contribution of the GDH pathway to ammonia assimilation in the cell, the affinity of GDH for NH_4^+ and the intracellular NH_4^+ concentration must be taken into account. In ammonia-grown cells, where the average intracellular NH_4^+ concentration was 13.8 mM, the activity of GDH with a K_m of 12.0 mM for NH_4^+ can be calculated to be 72 mU/mg of protein from the known activity at 25 mM NH_4^+ and the Michaelis-Menten equation. Comparison with the observed GS and GOGAT activities shows that GDH is an important pathway of ammonia assimilation in ammonia-grown cells. In N_2-fixing cells, the in vitro GDH activity measured at an NH_4^+ concentration of 25 mM (13.4 mU/mg of protein) was 2.5-fold higher than the GOGAT activity (5.4 mU/mg of protein). However, the average intracellular NH_4^+ concentration was 0.99 mM. At such an NH_4^+ concentration, the GDH activity can be calculated to be 11% of that at NH_4^+ concentration of 25 mM. Therefore, the actual rate of ammonia assimilation by the GDH pathway in N_2-fixing cells is expected to be $13.4 \times 0.11 = 1.5$ mU/mg of protein. Assuming that the GS and GOGAT of *C. kluyverii*, like those of other bacteria, have very low K_ms for their substrates and therefore can operate at the rates observed in the in vitro assay, 5 to 6 mU/mg of protein (Table 1), the

TABLE 1. Ammonia-assimilating enzymes in *C. kluyverii* and *C. butyricum* grown on different nitrogen sources

Species	Nitrogen source	Doubling time (h)	Intracellular NH_4^+ concn (mM)	Sp act (mU/mg of protein)					
				GDH		GS	GOGAT		G6PD
				NADPH	NADH		NADPH	NADH	
C. kluyverii	NH_4^+	19 ± 1[a]	13.8	91	ND[b]	23 ± 12	32	ND	
	N_2	60[c]	0.99	13.4	ND	5.9	5.4	ND	
C. butyricum	NH_4^+	2.4 ± 0.1[c]		ND	ND	20 ± 3	ND	110	
	N_2	15 ± 2[c]		ND	ND	27	ND	45	ND
	Complex			207 ± 20	ND				152

[a] Growth at 30°C.
[b] ND, Not detectable.
[c] Growth at 37°C.

contribution of the GDH pathway to ammonia assimilation appears to be minor compared with that of the GS-GOGAT pathway in N_2-fixing *C. kluyverii*.

***C. butyricum*. (i) Cells grown in glucose-salt medium with ammonia or N_2.** The cell extracts of *C. butyricum* growing exponentially in glucose-salt medium with NH_4^+ or N_2 as the nitrogen source were found to oxidize NADPH and NADH at the rates of 30 to 50 and 425 to 1,750 mU/mg of protein, respectively. The GDH and GOGAT activities were therefore assayed through observation of [^{15}N]glutamic acid formation. GDH activity was undetectable with NADPH or NADH as the coenzyme in either ammonia-grown or N_2-fixing cultures. The results of the GOGAT assay for ammonia-grown *C. butyricum* are shown in Fig. 3. [^{15}N]Glutamic acid was formed in the presence of NADH (Fig. 3A through D) but not in the presence of NADPH (Fig. 3E) or in the absence of coenzyme (Fig. 3F). The results show that the GOGAT of *C. butyricum* is NADH specific. Figure 4 shows the time-dependent formation of [^{15}N]glutamic acid. From the initial rates of formation, NADH-GOGAT activities of 110 and 45 mU/mg of protein were obtained for ammonia-grown and N_2-fixing *C. butyricum*, respectively. The specific activities of ammonia-assimilating enzymes summarized in Table 1 clearly show that the GS-GOGAT pathway is the predominant pathway of ammonia assimilation in both ammonia-grown and N_2-fixing *C. butyricum* in glucose-salt medium.

(ii) Cells grown in complex medium. When a 2% inoculum of *C. butyricum* growing exponentially in reinforced *Clostridium* medium was added to the nitrogen-free glucose-salts medium and grown under N_2 flow, the culture showed an effective growth of 80 Klett units in 17 h but showed no further growth in the following 9 h. Because the reinforced *Clostridium* medium is very rich in complex carbon and nitrogen sources (see Materials and Methods), the observed growth was probably due to complex nitrogen sources carried over from the inoculum and the cessation of growth due to the lag required for induction of nitrogenase on exhaustion of the combined nitrogen sources. Surprisingly, cell extracts prepared from cells harvested just before the cessation of growth had NADPH-GDH activity of 207 ± 20 mU/mg of protein. No NADH-dependent GDH activity was observed. The activity was measured by the spectrophotometric method because the background oxidations of NADPH and NADH in the absence of substrates were low, at 33 and 65 mU/mg of protein, respectively. The NADPH-GDH activity remained stable in the cell extract at −20°C for more than 5 months. The detection of GDH activity in *C. butyricum* grown on complex medium is in agreement with the report of Bachofen and Herr (2), who detected GDH

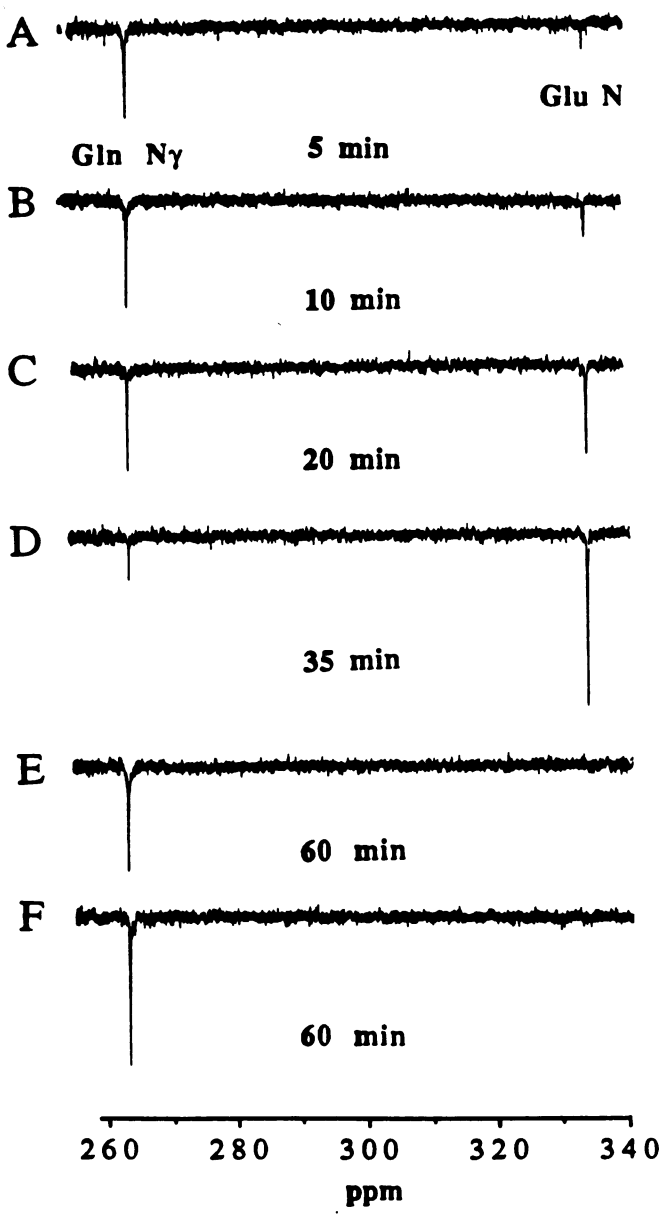

FIG. 3. ^{15}N NMR spectra of GOGAT assay solutions at various time intervals after addition of cell extracts of ammonia-grown *C. butyricum*. Assay preparations contained 7.5 mM NADH (A through D), 7.5 mM NADPH (E), or no coenzyme (F).

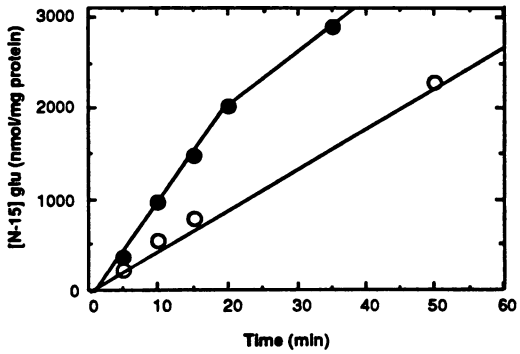

FIG. 4. Time-dependent formation of [^{15}N]glutamic acid in NADH-GOGAT assay solutions on addition of cell extracts of ammonia-grown (●) and N_2-fixing (○) *C. butyricum*.

activity in *C. butyricum* grown on complex nitrogen sources for 36 h (the growth phase and the specific activity of the enzyme were not reported). However, under their growth conditions, the GDH activity was higher with NADH than with NADPH.

The affinities of NADPH-GDH for NH_4^+ in the reductive amination reaction and for glutamate in the oxidative deamination reaction were measured to assess the physiological function of the enzyme. The Lineweaver-Burk plots of $1/v_0$ versus $1/[NH_4^+]$ were linear for NH_4^+ concentrations of 1 to 25 mM, with a K_m^{app} of 2.8 mM, but at higher (25 to 100 mM) NH_4^+ concentrations the slope of the least-squares line through the points was greater, with a K_m^{app} of 25.3 mM. Such biphasic kinetics with respect to NH_4^+ have been observed in GDHs from a number of organisms (33). With glutamate as the substrate, the plots were linear for glutamate concentrations of 5 to 50 mM with a K_m of 8.9 mM. The results suggest that the enzyme has an adequate affinity for NH_4^+ in the physiological NH_4^+ concentration range of 1 to 25 mM.

Attempts were made to better understand the growth conditions under which NADPH-GDH is derepressed. No NADPH-GDH activity was detected in *C. butyricum* growing exponentially in reinforced *Clostridium* medium, which is expected to be rich in complex nitrogen sources including glutamate. No GDH activity, NADPH or NADH dependent, could be detected in *C. butyricum* growing exponentially on the synthetic glucose-salt medium with 50 mM L-glutamate as the sole nitrogen source—a growth condition under which GDH with a catabolic function, if present, is expected to be maximally induced. These results suggest that the metabolic function of NADPH-GDH of *C. butyricum*, like that of most bacterial NADPH-specific GDHs, is to assimilate ammonia. Our results, in combination with that of Bachofen and Herr (2), suggest that derepression of NADPH-GDH in *C. butyricum* occurs in the presence of complex nutrients but not in the synthetic glucose-salt medium tested here. Further studies are needed to understand the factors that cause such derepression.

G6PD, an enzyme that catalyzes the first reaction in the pentose phosphate pathway and is responsible for formation of NADPH from $NADP^+$ in most organisms, has been reported to be undetectable in glucose-grown *C. butyricum* (12) (see Discussion). To investigate whether the absence of G6PD, which may result in an unusually low rate of regeneration of NADPH, can be one of the factors responsible for the absence of NADPH-dependent GDH activity in glucose-grown cells, the activity of G6PD was measured under various growth conditions. Cells fixing N_2 in glucose-salt medium had no G6PD activity (Table 1), in agreement with the reported result for glucose-grown cells (12). By contrast, the cells grown in the presence of complex carbon and nitrogen sources in addition to glucose under the conditions described above had a G6PD activity of 152 mU/mg of protein as well as an NADPH-GDH activity of 207 ± 20 mU/mg of protein. Thus, both enzymes are derepressed under this growth condition.

DISCUSSION

In *C. kluyverii* which has an NADPH-GDH with K_m^{app} for NH_4^+ of 12.0 mM, the GDH pathway was found to play a minor role relative to the GS-GOGAT pathway in ammonia assimilation during N_2 fixation. In this respect, *C. kluyverii* resembles *K. pneumoniae*, *Rhodospirillum rubrum*, and *Chromatium* sp. strain D, which have GDHs with high K_ms for NH_4^+ (6 to 16 mM) and utilize the GS-GOGAT pathway for ammonia assimilation during N_2 fixation (4, 5, 16, 17, 21).

In *C. butyricum* grown on synthetic glucose-salt medium with NH_4^+ or N_2 as the nitrogen source, the GDH activity was undetectable, and GS-GOGAT was the predominant pathway of ammonia assimilation. In this respect, *C. butyricum* resembles *C. pasteurianum* and *Azotobacter vinelandii*, which have undetectable levels of GDH even in ammonia-rich medium and assimilate ammonia by the GS-GOGAT pathway during N_2 fixation (3, 8, 17, 20, 21). However, *C. butyricum* is capable of synthesizing NADPH-GDH with a K_m^{app} for NH_4^+ of 2.8 mM, although the activity has so far been detected only after growth in complex media. Why is this enzyme repressed in *C. butyricum* during growth on glucose-salt media with NH_4^+ or N_2 as the nitrogen source? In *B. polymyxa* and *B. macerans*, NADPH-GDHs with comparable affinities for NH_4^+ are the major pathways of ammonia assimilation in both ammonia-grown and N_2-fixing cells (13, 14).

Two unusual characteristics of glucose fermentation in *C. butyricum* may have some bearing on this question. One is the apparent inability of glucose-grown *C. butyricum* to regenerate NADPH from $NADP^+$ directly through fermentation of glucose. The other is the presence of acetate and butyrate kinases in *C. butyricum*, which may allow efficient production of ATP from fermentation of glucose; as a result, the pool of ATP available for energy-requiring pathways such as the GS-GOGAT pathway may be somewhat higher in *C. butyricum* than in the *Bacillus* species during the energy-demanding process of nitrogen fixation.

The rate of regeneration of NADPH as a reductant for biosynthetic pathways may be unusually low in glucose-grown *C. butyricum*. Two key enzymes that are responsible for the regeneration of NADPH from $NADP^+$ in many other organisms, G6PD and the $NADP^+$-specific malic enzyme, have been reported as undetectable in glucose-grown *C. butyricum* and *C. pasteurianum* (12). The only known pathway by which NADPH is regenerated from $NADP^+$ in these two *Clostridium* species is through oxidation of NADH by NAD^+-ferredoxin oxidoreductase to form reduced ferredoxin, which in turn reduces $NADP^+$ to NADPH in a reaction catalyzed by $NADP^+$-ferredoxin oxidoreductase (12). NADH, on the other hand, is produced in abundance during the fermentation of glucose and has to be rapidly oxidized to allow glycolysis to proceed (7). Measurements of the intracellular concentrations of the two coenzymes in sucrose-grown *C. pasteurianum* showed the (NADPH + $NADP^+$)/(NADH + NAD^+) ratio to be very low, 0.024 (23).

In contrast, the $NADP^+/NAD^+$ ratio is 0.28 in glucose-grown *Bacillus licheniformis*, which has G6PD activity (22). These considerations suggest that a low rate of regeneration of NADPH resulting from the lack of G6PD activity may be one of the factors responsible for the absence of NADPH-GDH activity in ammonia-grown and N_2-fixing *C. butyricum* in glucose-salt medium. Furthermore, with excess NADH, which must be reoxidized to allow glycolysis to proceed, the alternative ammonia-assimilating pathway with an NADH-specific enzyme, the GS/NADH-GOGAT pathway, is expected to be more favorable in terms of efficient recycling of reductants. Significantly, in one of the growth media used in this study that contained complex carbon sources in addition to glucose, *C. butyricum* was found to have G6PD activity (Table 1) and thus is capable of regenerating NADPH from $NADP^+$ directly through oxidation of glucose. The same culture had high NADPH-GDH activity, suggesting that such a cellular environment may be more favorable for derepression and utilization of the NADPH-GDH pathway. It is hoped that future studies will lead to observation of the activities of these enzymes in *C. butyricum* growing in chemically defined media and thus permit investigation of the factors that regulate the synthesis of these enzymes.

In terms of ATP production, the fermentation pathways of *C. butyricum* may be more efficient than those of the N_2-fixing *Bacillus* species. *C. butyricum* ferments glucose to acetate and butyrate, yielding 3 to 4 mol of ATP per mol of glucose; the average is 3.3 mol of ATP per mol of glucose fermented in an ammonia-grown chemostat culture (7). *B. polymyxa* ferments glucose to ethanol and 2,3-butanediol in acidic media (32) and to ethanol and acetate in glucose-limited culture (1); the former pathway is expected to yield 2 mol of ATP per mol of glucose, and the latter is expected to yield 2 to 3 mol of ATP per mol of glucose depending on whether acetate kinase is present in *B. polymyxa* and on the ratio of ethanol/acetate formation. For obligate anaerobes such as *C. pasteurianum* and *C. butyricum*, development of high-energy yielding fermentation pathways was probably more crucial for survival than for facultative anaerobes such as the *Bacillus* species. If the efficiency of ATP production from fermentation of glucose is indeed significantly lower in *B. polymyxa* and *B. macerans* compared with that in the N_2-fixing *Clostridium* species, the drain on the ATP pool imposed by N_2 fixation can be more severe for the *Bacillus* species than has been observed in *C. pasteurianum* (30). Consequently, assimilation of ammonia through the GDH pathway without further expenditure of energy may well be more crucial to those N_2-fixing species having low energy-yielding fermentation pathways. We are currently investigating the efficiency of ATP production from fermentation of glucose as well as the levels of adenylate pools and ADP/ATP ratios in N_2-fixing *Bacillus* species to obtain a better understanding of the possible correlation between cellular energy state and ammonia-assimilating pathways in nitrogen-fixing bacteria.

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