

## Purification and Properties of NADP-Dependent Glutamate Dehydrogenase from *Ruminococcus flavefaciens* FD-1

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Glutamate dehydrogenase (GDH) (L-glutamate:NADP<sup>+</sup> oxidoreductase, deaminating, EC 1.4.1.4) from the cellulolytic ruminal bacterium *Ruminococcus flavefaciens* has been purified and characterized. The native enzyme and subunit are 280 and 48 kDa, respectively, suggesting that the native enzyme is a hexamer. The enzyme requires 0.5 M KCl for optimal activity and has a pH optimum of 6.9 to 7.0. The  $K_m$ s for ammonia,  $\alpha$ -ketoglutarate, and glutamate are 19, 0.41, and 62 mM, respectively. The sigmoidal NADPH saturation curve revealed positive cooperativity for the binding of this coenzyme. The first residue in the N-terminal amino acid sequence from *R. flavefaciens* GDH was alanine, suggesting that the protein may be modified posttranslationally. Comparison of the N-terminal sequence with those of *Escherichia coli*, *Salmonella typhimurium*, and *Clostridium symbiosum* revealed only 39% amino acid homologies. The GDH from *R. flavefaciens* was unique in that its specific activity was highest during ammonia-limited growth but was not affected by ammonia shock treatment (20 mM).

*Ruminococcus flavefaciens* is one of the predominant cellulolytic bacterial species of the rumen. Ammonia is its principal source of nitrogen, since urea (13), peptides, and amino acids (8) are not used effectively for growth of pure cultures. Therefore, the assimilation of ammonia is of fundamental importance to the contribution of this organism to ruminal degradation of fibrous feedstuffs.

The assimilation of ammonia has been thoroughly investigated with only a few rumen bacterial species (18, 30, 37), despite the observations from <sup>15</sup>N-labelling studies that 50 to 78% of ruminal bacterial nitrogen is derived from ammonia (25, 33) and that ammonia is indeed required by many ruminal bacteria (1, 8). In general, ammonia assimilatory enzymes similar to those of the enteric bacteria have been found in ruminal bacteria, although individual differences in optimum assay conditions and apparent regulatory behavior have been encountered previously (16, 43).

We have demonstrated the presence of NADP-dependent glutamate dehydrogenase (GDH) and glutamine synthetase in crude cell extracts of *R. flavefaciens* (14). In this communication, we report the purification and characterization of an NADP<sup>+</sup>-dependent glutamate dehydrogenase from *R. flavefaciens* FD-1.

### MATERIALS AND METHODS

**Organism and culture conditions.** *R. flavefaciens* FD-1 was obtained from the culture collection of the Department of Animal Sciences. Cultures were grown anaerobically by using a previously defined mineral medium (15) modified by the omission of 3-phenylpropionic acid and phenylacetic acid, the addition of 0.01% (wt/vol) sodium citrate, and the replacement of cysteine sulfide with 0.038% (wt/vol) sodium sulfide (Na<sub>2</sub>S) and 0.01% (wt/vol) dithiothreitol for batch cultures or 0.009% (wt/vol) Na<sub>2</sub>S and 0.015% (wt/vol) dithiothreitol for continuous cultures. Cellobiose (10 or 20 mM) was the carbon source, and NH<sub>4</sub>Cl (1, 2, or 10 mM) was the sole nitrogen source. Nutrient concentrations were selected

such that media were limiting in either nitrogen or carbon for cell growth. Anaerobic techniques (7, 17) were used for all manipulations involving media and cell preparations. Cells were cultured at 37°C.

For studies using crude extracts, cells were grown in batch cultures (1 to 2 liters until the late log phase of growth) or in continuous cultures (dilution rate, 0.14 h<sup>-1</sup>). A 10-liter ammonia-limited batch culture was used for the purification of GDH.

The abilities of cells to rapidly regulate their ammonia assimilatory enzymes were tested by the addition of a small volume (<1%, vol/vol) of sterile anaerobic water or concentrated NH<sub>4</sub>Cl solution to ammonia-limited cultures in the mid-log phase of growth or to continuously cultured cells collected after at least 4 culture volume turnovers. The final NH<sub>4</sub>Cl concentration in the ammonia-shocked treatments was 20 mM. The cells were incubated for 10 min at 37°C and then harvested as described below.

**Cell harvest and preparation of crude extract.** Batch cultures were harvested in the late log phase of growth, whereas continuous cultures were harvested either directly from the culture vessel or from 12-h collections in an effluent vessel kept in an ice bath. Cells were harvested by centrifugation (10,000 × g, 20 min, 4°C). The cell pellet was either frozen immediately at -70°C or washed once with anaerobic buffer (50 mM imidazole or bistrispropane, 1% KCl, 1 mM dithiothreitol, pH 6.8). For the preparation of cell extracts, washed cells were resuspended in 10 to 25 ml of this buffer (plus 50 μg of phenylmethylsulfonyl fluoride per ml) and passed twice through a chilled French pressure cell (20,000 lb/in<sup>2</sup>). Unbroken cells were removed by centrifugation (10,000 × g, 10 min, 4°C). Supernatants were then subjected to ultracentrifugation in a fixed angle rotor at 105,000 × g for 4 h (4°C), giving a membrane-free supernatant (S2) and a membrane-containing pellet (P2). In some experiments, the P2 pellet was washed by being resuspended in 3 ml of the buffer described above. The suspension was then subjected to ultracentrifugation under the same conditions as before to yield a P3 fraction.

**Enzyme assays.** GDH was assayed in the aminating direc-

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tion by using a modification (37) of the method of Meers et al. (27). Both NADH and NADPH were tested as coenzymes. Deaminating GDH was assayed in 0.5-ml reaction mixtures containing a buffer consisting of 0.5 M KCl, 1 mM NADP<sup>+</sup> or NAD<sup>+</sup>, and 50 mM imidazole, pH 7.0. Activity was defined by the disappearance (or the appearance, for the deaminating reaction) of NAD(P)H, measured by the  $A_{340}$ , upon the addition of the sample to the reaction mixture. Substrate saturation curves were determined for individual substrates, while others were present at enzyme-saturating concentrations. Kinetic parameters were estimated from computer-generated double-reciprocal ( $1/V$  versus  $1/S$ ) or Hill [ $\log [V/(V_{\max} - V)]$  versus  $\log S$ , where  $V$  is the observed velocity and  $V_{\max}$  is the maximal reaction velocity or rate] transformations of substrate saturation data.

**Enzyme purification.** GDH was purified by ion-exchange, affinity, and gel filtration chromatography. All separations were performed aerobically at room temperature, and fractions were refrigerated as soon as they were collected.

The S2 fraction was applied to a Mono Q HR 5/5 (Pharmacia, Piscataway, N.J.) anion-exchange column previously equilibrated with BD buffer (20 mM bistrispropane, 1 mM dithiothreitol, pH 6.8). After being washed with 5 ml of this buffer, bound proteins were eluted (0.5 ml/min) in a linear gradient (0 to 1 M) of NaCl. Proteins were detected by their  $A_{280}$  in a flowthrough cell (Dynamax UV-1; Rainin Instruments, Woburn, Mass.). Fractions (0.5 ml) were collected, and those with the highest GDH (aminating) activity were pooled and dialyzed overnight against 1 liter of BD buffer at 4°C.

Dialyzed GDH-containing fractions were then applied to a column containing 8 ml (2.0 g [dry weight]) of Red Sepharose CL-6B (Pharmacia) previously equilibrated with BDN buffer (BD buffer with 150 mM NaCl). Bound proteins were eluted (0.5 ml/min) in a linear gradient (0.15 to 3.00 M) of NaCl after the column had been washed with 4 volumes of BDN buffer. Fractions (1.0 ml) were collected, and GDH-containing fractions were again pooled. The pooled fractions were concentrated and desalted by using Centricon 10 centrifugal microconcentrators (Amicon, Danvers, Mass.).

A Superose 6 HR 10/30 gel filtration column (Pharmacia) was equilibrated with BD buffer at a flow rate of 0.3 ml/min. The concentrated GDH-containing fraction was then applied to the column. The fractions containing GDH were maintained separately for electrophoretic analysis.

**Electrophoresis.** Nondenaturing polyacrylamide gel electrophoresis (PAGE) was used to compare the migrations of GDH activity among crude cell fractions. The tube gels contained 3 and 7% polyacrylamide in the stacking and resolving gels, respectively. All other reagents, including the activity stain, were as described by Bellion and Tan (3). The electrophoresis was conducted in a Protean II unit (Bio-Rad, Richmond, Calif.) adapted for tube gels at 2 mA per tube (constant current) with cooling at 4°C.

The GDH-containing fractions from gel filtration chromatography were analyzed by sodium dodecyl sulfate (SDS)-PAGE (21) with a minigel apparatus (Mini Protean II; Bio-Rad). Acrylamide concentrations were 4 and 12% in stacking and resolving gels, respectively. The SDS-PAGE gels were silver stained as described by Merrill (28).

**Determination of native enzyme and subunit sizes.** The native size of purified GDH was determined by calibrating the gel filtration column with the following standard proteins: ferritin (450 kDa), catalase (240 kDa), aldolase (158 kDa), bovine serum albumin (68 kDa), hen egg albumin (45 kDa), and chymotrypsinogen A (25 kDa) (Combithek;

Boehringer Mannheim, Indianapolis, Ind.). The void volume of the column was determined with blue dextran (2,000 kDa).

The subunit size of GDH was determined by comparison with SDS-PAGE molecular weight standards (Bio-Rad) on the Coomassie blue-stained protein blot of an SDS-PAGE minigel (see below).

**Protein blotting and amino-terminal sequencing.** Proteins from an SDS-PAGE minigel were electroblotted onto polyvinylidene difluoride membranes (ProBlott; Applied Biosystems, Foster City, Calif.) by a wet-blotting procedure (Mini-TransBlot Electrophoretic Transfer Cell; Bio-Rad) according to the manufacturer's instructions. Blotted membranes were then stained for about 1 min with 0.1% (wt/vol) Coomassie brilliant blue R-250 in a solution containing 1% acetic acid and 40% methanol. Membranes were destained in successive washes of 50% methanol for a total of about 20 min.

The amino-terminal amino acid sequence was determined by automated microsequencing (Applied Biosystems) performed in the Protein Sequencing Laboratory of the Biotechnology Center at the University of Illinois at Urbana-Champaign.

**Other.** Protein concentrations in samples were determined by measurements of  $A_{230}$  and  $A_{260}$  (19). Ammonia nitrogen concentrations were determined by the method of Chaney and Marbach (10). Unless otherwise specified, all chemicals and reagents were purchased from Sigma.

## RESULTS

**Growth and expression of GDH activity.** *R. flavefaciens* FD-1 did not grow in media in which glutamate or methylamine was the sole nitrogen source and grew extremely slowly when provided with glutamine, apparently limited by the rate of spontaneous deamination of glutamine in solution. Ammonia supported growth, and nitrogen-limited (1 mM initially) batch cultures had higher GDH specific activities in the S2 fraction than carbon-limited (10 mM) cultures ( $3,315 \pm 289$  versus  $997 \pm 72$  nmol  $\cdot$  mg<sup>-1</sup>  $\cdot$  min<sup>-1</sup>, respectively). Residual ammonia concentrations in media were 7.50 and 0.08 mM for carbon- and nitrogen-limited cultures, respectively, after growth until the late log phase. Although GDH activity was higher under ammonia-limiting conditions, its activity in extracts from ammonia-limited cultures was not affected by sudden exposure of the cells to 20 mM NH<sub>4</sub>Cl. Exponential growth rates, as measured by optical densities (600 nm), were similar in ammonia- and cellobiose-limited cultures ( $0.28 \pm 0.02$  and  $0.31 \pm 0.04$  h<sup>-1</sup>, respectively).

Although GDH has been detected in the P2 fraction also, 78% of the total GDH activity (sum of the specific activity times protein concentration times volume for S2 and P2) in continuously cultured cells was found in S2 ( $164.0 \pm 5.7$  and  $47.4 \pm 6.2$   $\mu$ mol  $\cdot$  min<sup>-1</sup> in S2 and P2, respectively). The S1-, S2-, and P3-associated GDHs comigrated in nondenaturing polyacrylamide gels (Fig. 1). Activity stains of additional tube and slab gels which were subjected to electrophoresis for longer and shorter times and with different amounts of protein verified that only a single band of GDH activity existed in crude extracts (data not shown). These results suggested that only one form of the enzyme exists. GDH was subsequently purified 119-fold from the S2 fraction of cells from an ammonia-limited batch culture (Table 1; Fig. 2).

Two major proteins (48 and 37 kDa) eluted close to each other from the gel filtration column (Fig. 3), as observed on

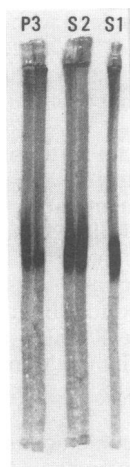


FIG. 1. Comigration of GDH activity from crude cell fractions S1, S2, and P3 in nondenaturing PAGE. The tube gels were loaded with 40  $\mu\text{g}$  of protein from the respective fractions and stained for GDH activity following electrophoresis.

the SDS-PAGE gel. The 48-kDa protein and the GDH activity began to elute before the 37-kDa protein appeared. The GDH activity peak corresponded with the peak of the 48-kDa, but not that of the 37-kDa, protein. Therefore, fractions with GDH activity but without the 37-kDa protein were pooled. Two minor bands of protein (76 and 85 kDa) were still detected in the purified GDH fraction, as seen in the Coomassie blue-stained protein blot of an SDS-PAGE gel (Fig. 4). These contaminating bands were estimated to compose 4% of the total protein in the fraction by using integrations of peaks from scanning laser densitometry.

The size of the native GDH enzyme was estimated to be 280 kDa with standard molecular size markers by gel filtration chromatography. The subunit size, estimated from standard size markers on the SDS-PAGE gel, was 48 kDa. The formation of a hexamer in the native enzyme would be consistent with this evidence.

The purified GDH was specific for NADPH, and no NADH-linked activity was detected. The specific activity of the purified enzyme increased with temperature to an optimum of 46°C. However, assays to characterize GDH were conducted at the more physiological temperature of 37°C. The optimal KCl concentration in the reaction mixture was 0.5 M; NaCl could replace KCl at this level. The enzyme

TABLE 1. Purification of GDH from *R. flavofaciens* FD-1

Step	Total activity (U) <sup>a</sup>	Total protein (mg)	Sp act (U · mg <sup>-1</sup> )	Purification (fold)	Yield (%)
S2	271,495	126.54	2,146	1	100
Mono Q	244,896	21.15	11,579	5	90
Red Sepharose	217,465	4.32	51,532	24	80
Superose 6	94,787	0.37	256,180	119	35

<sup>a</sup> Units were determined as nanomoles per minute.

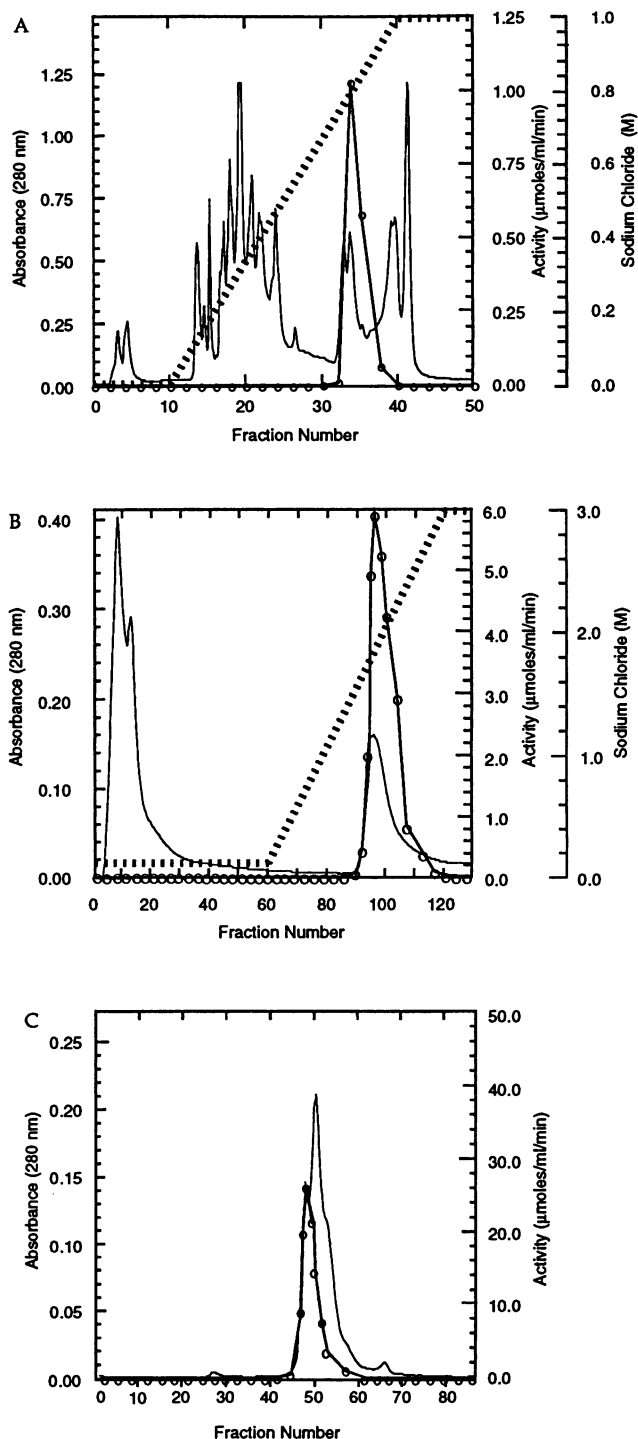


FIG. 2. Chromatographic profiles of the GDH purification steps Mono Q ion exchange (A), Red Sepharose affinity chromatography (B), and Superose 6 gel filtration (C). Absorbances (—), NaCl concentration gradient (· · · ·), and activities (○) are indicated.

displayed a broad pH optimum for the aminating reaction, with a peak at pH 6.9 to 7.0.

Typical saturation kinetics were observed for most substrates with the purified enzyme. The  $K_m$ s for substrates are shown in Table 2, along with values for some other purified



FIG. 3. Gel filtration fractions showing major bands corresponding to GDH activity and a contaminating protein. The minigel was silver stained (25). Lane 1 contained the following protein molecular size markers (in kilodaltons) (from top): myosin, 200;  $\beta$ -galactosidase, 116; bovine serum albumin, 66; ovalbumin, 45; and carbonic anhydrase, 31. Lanes 2 to 7 contained 5  $\mu$ l (0.33 to 1.03  $\mu$ g of protein) of gel filtration fractions 46 to 51, respectively, with the corresponding GDH activities (in micromoles per milliliter per minute): 9.2, 20.0, 26.0, 21.8, 15.2, and 8.0.



FIG. 4. SDS-PAGE of the purified GDH from *R. flavefaciens* FD-1. The proteins on the minigel were blotted on a PVDF membrane and stained with Coomassie brilliant blue R-250. The left lane contained the following protein molecular size markers (in kilodaltons) (from top): myosin, 200;  $\beta$ -galactosidase, 116; phosphorylase b, 97; bovine serum albumin, 66; ovalbumin, 45; carbonic anhydrase, 31; and soybean trypsin inhibitor, 21.5. The right lane contained the purified GDH (5  $\mu$ g of protein).

GDHs. The saturation curve for NADPH in the aminating reaction had a slightly sigmoidal shape. The Hill coefficient had a value of 1.96, and the Hill equation predicted a substrate concentration of 34  $\mu$ M at one-half the observed maximum rate of reaction.

The N-terminal amino acid sequence (30 residues) (Table 3) of the GDH subunit was determined in order to identify probe sequences for subsequent genetic studies. The N-terminal amino acid was alanine rather than methionine. The ungapped N-terminal sequences from *R. flavefaciens* FD-1, *Escherichia coli* (26, 40), and *Salmonella typhimurium* (2) were identical at 12 of the first 30 positions. The gapped and aligned N-terminal sequences of *R. flavefaciens* FD-1 and *Clostridium symbiosum* (NAD<sup>+</sup>-specific GDH) (22) were also identical at 12 positions. The gapped and aligned sequences of these four species revealed 7 positions with identical amino acids (Table 3).

## DISCUSSION

The ruminal environment is characterized by its anaerobicity, constant temperature (37 to 39°C), moderate levels of salts (100 to 200 mM) and ammonia (~5 to 30 mM), and buffering at nearly neutral pH (pH 6.5 to 7.5). The optimal pH and salt levels for GDH from *R. flavefaciens* FD-1 reflect this environment. Similar salt requirements for optimal GDH

activity have been reported in studies with *Selenomonas ruminantium* (37) and *Succinivibrio dextrinosolvens* (30). Caldwell and Hudson (9) demonstrated that the predominant ruminal bacteria require 100 to 200 mM sodium for growth.

The specific activity of GDH in crude extracts of batch-cultured *R. flavefaciens* was highest when ammonia was limiting in media. Similar results were obtained from continuous cultures (31). Since GDH activity was unaffected by a sudden exposure of ammonia-limited cultures to 20 mM NH<sub>4</sub>Cl, posttranslational regulation of GDH by ammonia levels is unlikely.

In other ruminal bacteria studied to date, GDH specific activities were typically lower when cultures were ammonia limited than when they were carbon limited (18, 30, 37). The enteric bacteria *Klebsiella aerogenes* and *E. coli* repress GDH synthesis when ammonia is limiting or when they are grown with glutamate, and they rely on high-affinity glutamine synthetase coupled with glutamate synthase for ammonia assimilation and net glutamate formation (6, 27, 42). *S. typhimurium*, on the other hand, does not repress GDH synthesis under these conditions (5). Some of the bacilli synthesize GDH when grown with NH<sub>4</sub>Cl but not when grown with glutamate, while others lack biosynthetic GDH altogether (32).

In contrast, the intestinal anaerobe *Bacteroides fragilis*

TABLE 2. Comparison of native and subunit sizes and kinetic parameters among purified or partially purified GDHs from bacteria

Organism	Size (kDa)		$K_m$ (mM)					Reference(s)
	Native	Subunit	NH <sub>3</sub>	$\alpha$ kg <sup>a</sup>	glu <sup>b</sup>	NADPH	NADP <sup>+</sup>	
<i>R. flavefaciens</i> FD-1	280	48	19.22	0.41	62.19	0.034 <sup>c</sup>	ND <sup>d</sup>	This paper
<i>Escherichia coli</i> B/r	300	50	1.1	0.64	1.3	0.04	0.042	36
<i>Escherichia coli</i> D <sub>5</sub> H <sub>3</sub> G <sub>7</sub>	275	45	36	3.25	2.2	0.083	0.11	24
<i>Salmonella typhimurium</i> LT2	280	45	0.29	4.0	50.0	0.019	0.013	2, 11, 12
<i>Bacteroides fragilis</i> ATCC 23745	290	49	1.25	0.20	3.83	0.013	0.029	35
<i>Bacillus licheniformis</i> A-5	310	50	5.5	6.7	39	0.12	NR <sup>d</sup>	4, 32
<i>Clostridium</i> strain SB4 <sup>e</sup>	275	NR <sup>d</sup>	0.32	0.65	1.8	0.01	0.01	44
<i>Streptomyces fradiae</i> 30/3	200	49	30.8	1.54	28.6	0.07	0.12	41
<i>Pseudomonas</i> sp. strain AM1	190	50	20.2	0.76	31.6	0.033	NR <sup>d</sup>	3

<sup>a</sup>  $\alpha$ kg,  $\alpha$ -ketoglutarate.

<sup>b</sup> glu, glutamate.

<sup>c</sup> This value represents [NADPH] at 0.5  $V_{max}$  and is calculated from the Hill equation. Because of cooperative binding of NADPH, this value is mathematically distinct from  $K_m$ .

<sup>d</sup> ND, not determined; NR, not reported.

<sup>e</sup> This GDH is NAD<sup>+</sup> specific; therefore,  $K_m$ s for coenzyme refer to the unphosphorylated forms NADH and NAD<sup>+</sup>.

TABLE 3. Comparison of the N-terminal amino acid sequence determined for GDH from *R. flavefaciens* with other corresponding sequences

Organism (reference[s])	Amino acid sequence
<i>R. flavefaciens</i> FD-1.....	ALKNQYLKELLERV EKRNPGPEPEFIQAVIE
<i>C. symbiosum</i> (22).....	SKY VDRVI AEVEKKYADEPEFVQIVVE
<i>E. coli</i> (26, 40).....	MDQTYSLSEFLNHV QKRDPNQTEFAQAVRE
<i>S. typhimurium</i> (2).....	MDQTCSLSEFLNHV QKRDPHQTEFAQAVRE

possesses about twice as much immunoprecipitable GDH, as a percentage of total protein, and 10-fold-higher GDH specific activity when grown in ammonia-limiting rather than ammonia-sufficient media (45). The high-activity form was rapidly inactivated by the exposure of cells to 50 mM  $\text{NH}_4\text{Cl}$ . The inactivation was reversible by transferring cells to ammonia-limited medium. The reversible inactivation mechanism did not require protein synthesis and did not involve phosphorylation of GDH. In contrast, phosphorylation has been previously demonstrated with and may be a regulatory feature of *E. coli* GDH (23).

The native and subunit sizes of GDH purified from *R. flavefaciens* are consistent with the formation of a hexameric protein, similar to the enzymes purified from several other species of bacteria (Table 2). *Pseudomonas* sp. strain AM1 (3) and *Streptomyces fradiae* (41) have subunits comparable to those of these other bacteria in size, but native sizes of 190 to 200 kDa suggest a tetrameric structure.

Kinetic constants for the substrates of purified,  $\text{NADP}^+$ -dependent GDH from *R. flavefaciens* FD-1 are within the range of those determined for other species of bacteria (Table 2). In general, the high affinity for  $\alpha$ -ketoglutarate and the low affinity for glutamate suggest that the reductive amination of  $\alpha$ -ketoglutarate is the primary role of this enzyme. Affinities for ammonia vary considerably among organisms and even among strains of *E. coli*. The apparent  $K_m$  for ammonia in the ruminal bacterium *Ruminobacter (Bacteroides) amylophilus* was 1 to 2 mM (18); two values, 6.7 and 23 mM, were determined for low and high concentration ranges in assays using *S. ruminantium* (37).

The GDH from *R. flavefaciens* exhibited positive cooperativity (Hill coefficient, 1.96) for NADPH but not for the other substrates. Allosteric has been shown for GDH from bovine liver (38) but to our knowledge has not been reported for any substrates of bacterial  $\text{NADP}^+$ -specific GDHs. Positive cooperativity in ligand binding implies that the affinity for the ligand increases after the first molecule(s) is bound. The change in affinity is commonly associated with a conformational change in the enzyme. Evidence for conformational differences in  $\text{NAD}^+$ -dependent GDH from *C. symbiosum* when it is crystallized in the presence or absence of glutamate was previously reported, but effects due to coenzyme binding were not addressed (39). The physiological significance of the cooperative binding of NADPH by GDH from *R. flavefaciens* FD-1 may be to allow fine control in balancing available biosynthetic reducing power with flux through the glutamate pool. Such control may be especially important in an organism which utilizes GDH as the primary means of assimilating its sole N source, ammonia.

*R. flavefaciens* grew in both continuous and batch cultures when the prevailing ammonia concentration was below 100  $\mu\text{M}$ . Under these conditions, GDH specific activity was maximal. It is interesting that an enzyme with such a low affinity for ammonia would be induced as ammonia became limiting. It is feasible that the cell maintains a higher internal

concentration of ammonia by using an active transport, or recycling, system. However, we could not demonstrate ammonium transport in ammonia-starved cells with the radioactive analog [ $^{14}\text{C}$ ]methylamine.

Russell and Strobel (34) have measured ammonia gradients (the intracellular ammonia concentration/extracellular ammonia concentration ratio) in mixed ruminal bacteria but not in individual species. Their data showed only 15-fold gradients at most, which would still leave the intracellular ammonia concentration at 1/10 the  $K_m$  for GDH of *R. flavefaciens* in an ammonia-limited (<0.1 mM) culture. While some cyanobacteria may reach concentration gradients of >1,000-fold, most gradients achieved by bacterial species reviewed by Kleiner (20) ranged from 10- to 120-fold. Further study of ammonium gradients across the cell membrane of *R. flavefaciens* grown under different conditions should help to clarify how GDH contributes to the cellular nitrogen economy.

The N-terminal amino acid sequence of *R. flavefaciens* GDH begins with alanine, which suggests that this protein might be modified posttranslationally. Although the N-terminal amino acid in the GDH of *C. symbiosum* is serine, it is methionine in those of *E. coli* and *S. typhimurium* (Table 3). Homologies described in the N-terminal amino acid sequences of *R. flavefaciens* and other species suggest conservations of sequence among certain GDHs. However, these N-terminal homologies are not located in the conserved regions associated with pyridine nucleotide binding or catalytic function (29).

#### REFERENCES

- Allison, M. J. 1969. Biosynthesis of amino acids by ruminal microorganisms. *J. Anim. Sci.* **29**:797-807.
- Bansal, A., M. A. Dayton, H. Zalkin, and R. F. Colman. 1989. Affinity labelling of a glutamyl peptide in the coenzyme binding site of NADP-specific glutamate dehydrogenase of *Salmonella typhimurium* by 2-[(4-bromo-2,3-dioxobutyl)thio]-1, $\text{N}^6$ -ethenoadenosine 2',5'-bisphosphate. *J. Biol. Chem.* **264**:9827-9835.
- Bellion, E., and F. Tan. 1984. NADP-dependent glutamate dehydrogenase from a facultative methylotroph, *Pseudomonas* sp. strain AM1. *J. Bacteriol.* **157**:435-439.
- Bernlohr, R. W., H. J. Schreier, and T. J. Donahue. 1984. Enzymes of glutamate and glutamine biosynthesis in *Bacillus licheniformis*. *Curr. Top. Cell. Reg.* **24**:145-152.
- Brenchley, J. E., C. A. Baker, and L. G. Patil. 1975. Regulation of the ammonia assimilatory enzymes in *Salmonella typhimurium*. *J. Bacteriol.* **124**:182-189.
- Brenchley, J. E., M. Prival, and B. Magasanik. 1973. Regulation of the synthesis of enzymes responsible for glutamate formation in *Klebsiella aerogenes*. *J. Biol. Chem.* **248**:6122-6128.
- Bryant, M. P. 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. *Am. J. Clin. Nutr.* **25**:1324-1328.
- Bryant, M. P., and I. M. Robinson. 1963. Apparent incorporation of ammonia and amino acid carbon during growth of selected species of ruminal bacteria. *J. Dairy Sci.* **46**:150-154.
- Caldwell, D. R., and R. F. Hudson. 1974. Sodium, an obligate growth requirement for predominant rumen bacteria. *Appl. Microbiol.* **27**:549-552.
- Chaney, A. L., and E. P. Marbach. 1962. Modified reagents for determination of urea and ammonia. *J. Clin. Chem.* **8**:130-132.
- Coulton, J. W., and M. Kapoor. 1973. Purification and some properties of the glutamate dehydrogenase of *Salmonella typhimurium*. *Can. J. Microbiol.* **19**:427-438.
- Coulton, J. W., and M. Kapoor. 1973. Studies on the kinetics and regulation of glutamate dehydrogenase of *Salmonella typhimurium*. *Can. J. Microbiol.* **19**:439-450.
- Duncan, P. A., and R. I. Mackie. Unpublished data.
- Duncan, P. A., B. A. White, and R. I. Mackie. 1991. Partial characterization of ammonia assimilatory enzymes of *Ruminococcus flavefaciens* FD-1, abstr. K-92, p. 229. Abstr. 91st Gen.

- Meet. Am. Soc. Microbiol. 1991. American Society for Microbiology, Washington, D.C.
15. Helaszek, C. T., and B. W. White. 1991. Cellobiose uptake and metabolism by *Ruminococcus flavefaciens*. *Appl. Environ. Microbiol.* **57**:64-68.
  16. Hespell, R. B. 1984. Influence of ammonia assimilation pathways and survival strategy on ruminal microbial growth, p. 346-358. *In* F. M. C. Gilchrist and R. I. Mackie (ed.), *Herbivore nutrition in the subtropics and tropics*. The Science Press, Johannesburg, South Africa.
  17. Hungate, R. E. 1969. A roll tube method for cultivation of strict anaerobes, p. 117-132. *In* J. R. Norris and E. W. Ribbons (ed.), *Methods in microbiology*, vol. 3. Academic Press, Inc., New York.
  18. Jenkinson, H. F., P. J. Buttery, and D. Lewis. 1979. Assimilation of ammonia by *Bacteroides amylophilus* in chemostat cultures. *J. Gen. Microbiol.* **113**:305-313.
  19. Kalb, V. F., Jr., and R. W. Bernlohr. 1977. A new spectrophotometric assay for protein in cell extracts. *Anal. Biochem.* **82**:362-371.
  20. Kleiner, D. 1985. Bacterial ammonium transport. *FEMS Microbiol. Rev.* **32**:87-100.
  21. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
  22. Lilley, K. S., P. J. Baker, K. L. Britton, T. J. Stillman, P. E. Brown, A. J. G. Moir, P. C. Engel, D. W. Rice, J. Ellis-Bell, and E. Bell. 1991. The partial amino acid sequence of the NAD<sup>+</sup>-dependent glutamate dehydrogenase of *Clostridium symbiosum*: implications for the evolution and structural basis of coenzyme specificity. *Biochim. Biophys. Acta* **1080**:191-197.
  23. Lin, H.-P. P., and H. C. Reeves. 1991. Phosphorylation of *Escherichia coli* NADP<sup>+</sup>-specific glutamate dehydrogenase. *Curr. Microbiol.* **22**:181-184.
  24. Lin, H.-P. P., and H. C. Reeves. 1991. Purification and characterization of NADP<sup>+</sup>-specific glutamate dehydrogenase from *Escherichia coli*. *Curr. Microbiol.* **22**:371-376.
  25. Mathison, G. W., and L. P. Milligan. 1971. Nitrogen metabolism in sheep. *Br. J. Nutr.* **25**:351-366.
  26. McPherson, M. J., and J. C. Wootton. 1983. Complete nucleotide sequence of the *Escherichia coli* *gdhA* gene. *Nucleic Acids Res.* **11**:5257-5266.
  27. Meers, J. L., D. W. Tempest, and G. M. Brown. 1970. Glutamine (amide):2 oxoglutarate amino transferase (NADP), an enzyme involved in the synthesis of glutamate by some bacteria. *J. Gen. Microbiol.* **64**:187-194.
  28. Merrill, C. R. 1981. Ultra-sensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* **211**:1437-1438.
  29. Nagata, S., K. Tanizawa, N. Esaki, Y. Sakamoto, H. Tanaka, and K. Soda. 1988. Gene cloning and sequence determination of leucine dehydrogenase from *Bacillus stearothermophilus* and structural comparison with other NAD(P)<sup>+</sup>-dependent dehydrogenases. *Biochemistry* **27**:9056-9062.
  30. Patterson, J. A., and R. B. Hespell. 1985. Glutamine synthetase activity in the ruminal bacterium *Succinivibrio dextrinosolvens*. *Appl. Environ. Microbiol.* **50**:1014-1020.
  31. Pettipher, G. L., and M. J. Latham. 1979. Characteristics of enzymes produced by *Ruminococcus flavefaciens* which degrade plant cell walls. *J. Gen. Microbiol.* **110**:21-27.
  32. Phibbs, P. V., and R. W. Bernlohr. 1971. Purification, properties, and the regulation of glutamic dehydrogenase of *Bacillus licheniformis*. *J. Bacteriol.* **106**:375-385.
  33. Pilgrim, A. F., F. V. Gray, R. A. Weller, and C. B. Belling. 1970. Synthesis of microbial protein from ammonia in the sheep's rumen and the proportion of dietary nitrogen converted into microbial nitrogen. *Br. J. Nutr.* **24**:589-598.
  34. Russell, J. B., and H. B. Strobel. 1987. Concentration of ammonia across cell membranes of mixed ruminal bacteria. *J. Dairy Sci.* **70**:970-976.
  35. Saito, H., I. Yamamoto, and M. Ishimoto. 1988. Reversible inactivation of glutamate dehydrogenase in *Bacteroides fragilis*: purification and characterization of high activity- and low activity-enzymes. *J. Gen. Appl. Microbiol.* **34**:377-385.
  36. Sakamoto, N., A. M. Kotre, and M. A. Savageu. 1975. Glutamate dehydrogenase from *Escherichia coli*: purification and properties. *J. Bacteriol.* **124**:775-783.
  37. Smith, C. J., R. B. Hespell, and M. P. Bryant. 1980. Ammonia assimilation and glutamate formation in the anaerobe *Selenomonas ruminantium*. *J. Bacteriol.* **141**:593-602.
  38. Smith, E. L., B. M. Austen, K. M. Blumenthal, and J. F. Nyc. 1975. Glutamate dehydrogenases, p. 293-367. *In* P. D. Boyer (ed.), *The enzymes*, vol. XI. Academic Press, New York.
  39. Stillman, T. J., P. J. Baker, K. L. Britton, D. W. Rice, and H. F. Rodgers. 1992. Effect of additives on the crystallization of glutamate dehydrogenase from *Clostridium symbiosum*: evidence for a ligand-induced conformational change. *J. Mol. Biol.* **224**:1181-1184.
  40. Valle, F., B. Becerril, E. Chen, P. Seeburg, H. Heyneker, and F. Bolivar. 1984. Complete nucleotide sequence of the glutamate dehydrogenase gene from *Escherichia coli* K-12. *Gene* **27**:193-199.
  41. Vancurová, I., A. Vancura, J. Volc, J. Kopecky, J. Neuzil, G. Basarová, and V. Behal. 1989. Purification and properties of NADP-dependent glutamate dehydrogenase from *Streptomyces fradiae*. *J. Gen. Microbiol.* **135**:3311-3318.
  42. Varricchio, F. 1969. Control of glutamate dehydrogenase synthesis in *Escherichia coli*. *Biochem. Biophys. Acta* **177**:560-564.
  43. Wallace, R. J., and M. A. Cotta. 1988. Metabolism of nitrogen-containing compounds, p. 217-249. *In* P. N. Hobson (ed.), *The rumen microbial ecosystem*. Elsevier Applied Science, New York.
  44. Winnacker, E. L., and H. A. Barker. 1970. Purification and properties of an NAD-dependent glutamate dehydrogenase from *Clostridium* SB<sub>4</sub>. *Biochim. Biophys. Acta* **212**:225-242.
  45. Yamamoto, I., H. Saito, and M. Ishimoto. 1987. Regulation of synthesis and reversible inactivation *in vivo* of dual coenzyme-specific glutamate dehydrogenase in *Bacteroides fragilis*. *J. Gen. Microbiol.* **133**:2773-2780.