Role of NAD-Linked Glutamate Dehydrogenase in Nitrogen Metabolism in *Saccharomyces cerevisiae*

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We cloned *GDH2*, the gene that encodes the NAD-linked glutamate dehydrogenase in the yeast *Saccharomyces cerevisiae*, by purifying the enzyme, making polyclonal antibodies to it, and using the antibodies to screen a λgt11 yeast genomic library. A yeast strain with a deletion-disruption allele of *GDH2* which replaced the wild-type gene grew very poorly with glutamate as a nitrogen source, but growth improved significantly when the strain was also provided with adenine or other nitrogenous compounds whose biosynthesis requires glutamine. Our results indicate that the NAD-linked glutamate dehydrogenase catalyzes the major, but not sole, pathway for generation of ammonia from glutamate. We also isolated yeast mutants that lacked glutamate synthase activity and present evidence which shows that normally NAD-linked glutamate dehydrogenase is not involved in glutamate biosynthesis, but that if the enzyme is overexpressed, it may function reversibly in intact cells.

The existence of an NAD-linked glutamate dehydrogenase (NAD-GDH) in cells of *Saccharomyces cerevisiae* was first reported by Holzer and Schneider more than 30 years ago (12), but the role of this enzyme in glutamate metabolism is still unclear. Although mutations that lower the activity of this enzyme (*gdhCS* mutations) have been described, it has not been determined whether these mutations are in the structural gene for NAD-GDH or affect the regulation of this gene (28). NAD-GDH catalyzes the NAD-dependent oxidative deamination of glutamate to yield α-ketoglutarate and ammonia, as follows:

$$\text{Glutamate} + \text{NAD}^+ \rightarrow \text{α-ketoglutarate} + \text{NH}_4^+ + \text{NADH}$$

Although this reaction is reversible, two lines of circumstantial evidence suggest that in intact cells NAD-GDH is chiefly a catabolic enzyme. First, under normal growth conditions the concentration of NAD^+ in the cytoplasm is much greater than the concentration of NADH, forcing the equilibrium of the reaction toward degradation of glutamate (18). Second, NAD-GDH levels in cells grown with glutamate as a source of nitrogen are much higher than NAD-GDH levels in cells grown with ammonia as a source of nitrogen (3, 5–7, 9, 11, 21). Regulation of this reaction is dependent upon the products of at least two genes, *URE2* and *GLN3*; in *ure2* strains NAD-GDH activity is always high, in *gln3* strains NAD-GDH activity is low irrespective of the nitrogen source, and *gln3* is epistatic to *ure2* (3, 5, 9, 21).

Evidence for an anabolic role for NAD-GDH has also been reported. The NADP-linked glutamate dehydrogenase (NADP-GDH) catalyzes the same reversible reaction as NAD-GDH, except that it utilizes NADP and NADPH as the electron acceptor and donor, respectively. Grenson et al. found that *S. cerevisiae* mutants that lacked NADP-GDH grew slowly with ammonia as a nitrogen source, indicating that this enzyme is involved in glutamate biosynthesis (9). In addition, these investigators isolated second-site revertants of these mutants which grew normally with ammonia as a nitrogen source and found that the new mutation was allelic to *ure2*. Since NAD-GDH is derepressed in *ure2* strains

grown with ammonia, the implication is that NAD-GDH plays a role in glutamate biosynthesis and that this enzyme might be responsible for the residual growth on ammonia of strains lacking NADP-GDH. However, another enzyme that is capable of synthesizing glutamate, glutamate synthase, has also been found in *S. cerevisiae* (24). Glutamate synthase catalyzes the reductive amination of α-ketoglutarate by glutamine and NADH to yield two molecules of glutamate, as follows:

$$\text{α-Ketoglutarate} + \text{glutamine} + \text{NADH} \rightarrow 2 \text{ glutamate} + \text{NAD}^+$$
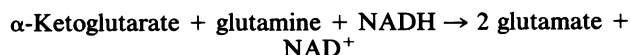
Starting with strains that lacked NADP-GDH, Grenson et al. (9) and Folch et al. (8) isolated double mutants (*gdh1 ama* or *gdh1 gus*) that were severely impaired in the ability to use ammonia as a nitrogen source. These mutants were shown to lack glutamate synthase activity (8, 28). This suggests that if NAD-GDH plays a role in glutamate synthesis in strains that lack NADP-GDH, it must share this role with glutamate synthase.

In the fungus *Aspergillus nidulans*, mutants that lacked NAD-GDH were obtained by screening for strains that were unable to grow with glutamate as a sole nitrogen source (15). We set out to isolate *S. cerevisiae* mutants which lack NAD-GDH by using a similar screening procedure, but were unable to find a mutant which had this growth phenotype and lacked the enzyme. Therefore, we cloned the *GDH2* gene by screening a λgt11 library with antibody made to purified yeast NAD-GDH. We used the clone to replace the genomic gene with a null allele to analyze the role of NAD-GDH in glutamate metabolism. Our results show that NAD-GDH enables cells to obtain the ammonia required for the synthesis of glutamine from glutamate.

MATERIALS AND METHODS

Yeast growth and genetic techniques. Mutagenesis of *S. cerevisiae* strains was carried out by using a previously described method (A. Mitchell, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1984), with minor modifications. Cells from stationary-phase cultures were collected, washed with buffer (0.1 M NaPO_4, pH 7), suspended

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TABLE 1. Yeast and bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source
Yeast strains		
AM736-12a	*MATa ade2-102 ura3-52 gln3-1 can1*	A. Mitchell
BC34	*MATa ade1-100 gdh1-6*	W. Courchesne
BC34-9	*MATa ade1-100 gdh1-6 glt1-2*	This study
BC34-10	*MATa ade1-100 gdh1-6 glt1-1*	This study
BC61	*MATα ure2-1*	W. Courchesne
MB1000	*MATα*	M. Brandriss
P6-1a	*MATa ura3-52 gln3-1*	P. Coschigano
P24-000	*MATα ade2-102 ura3-52 leu2-3,112 HIS4*	P. Coschigano
	MATa ADE2 URA3 leu2-3,112 his4-619	
PM35	*MATa ade2-102 leu2-3,112 gdh1-6*	P. Minehart
PM38	*MATα ura3-52 leu2-3,112*	P. Minehart
PM44	*MATα ade2-102 ura3-52 leu2-3,112 gdh1-6 gln3Δ4::LEU2*	P. Minehart
SM10-2a	*MATa ade2-102 gdh1-6*	This study
SM10-2a-150	*MATa ade2-102 gdh1-6 gdh2-1*	This study
SM14-5d	*MATα ura3-52*	This study
SM50-1a	*MATα ade1-100*	This study
SM50-1b	*MATα ura24 gdh1-6*	This study
SM50-1c	*MATα ura24 glt1-2*	This study
SM50-2c	*MATα gdh1-6 glt1-1*	This study
SM77-2b	*MATa gdh1-6*	This study
SM77-2c	*MATα ura3-52*	This study
SM77-3b	*MATα ade1-100 ura3-52 glt1-1*	This study
SM77-3c	*MATa gdh1-6 glt1-1*	This study
SM88-2a	*MATa ura3-52 gdh2-1*	This study
SM88-2d	*MATα ura3-52*	This study
SM94-4a	*MATα ura3-52 leu2-3,112 gdh2Δ2::LEU2*	This study
SM94-4b	*MATα his4-619*	This study
SM123-6b	*MATa ura3-52 leu2-3,112 his4-619 gdh1-6 glt1-1*	This study
SM124-1c	*MATα leu2-3,112*	This study
SM124-2d	*MATα leu2-3,112 gdh2Δ2::LEU2*	This study
SM1	*MATa ade2-102 leu2-3,112 gdh1-6 gdh2Δ2::LEU2*	This study
SM2	PM35 (YEp13)	This study
SM3	SM123-6b (YEp13)	This study
SM4	SM3 (pSM7)	This study
SM5	PM44 (pSM8)	This study
SM6	PM44 (YEp24)	This study
Bacterial strains		
Y1089	*E. coli ΔlacU169 proA⁺ Δlon araD139 strA hflA150 [chr::Tn10] (pBR322-lacIᵠ)*	R. Young
Y1090	*E. coli ΔlacU169 proA⁺ Δlon araD139 strA supF [trpC22::Tn10] (pBR322-lacIᵠ)*	R. Young
Plasmids		
pSM0	12-kb yeast genomic fragment containing part of the *GDH2* gene in YCp50	This study
pSM1	11-kb yeast genomic fragment containing the *GDH2* gene in YCp50	This study
pSM2	6.6-kb *SmaI-EagI* fragment from pSM1 in YCp50	This study
pSM3	4.3-kb *SalI-SalI* (partial) fragment from pSM1 in YCp50	This study
pSM4	2.3-kb *ClaI-ClaI* fragment from pSM1 in YIp5	This study
pSM5	5.0-kb *SmaI-ClaI* fragment from pSM1 in YIp5	This study
pSM6	2.2-kb *SalI-XhoI* fragment containing *LEU2* (from YEp13) replacing the 2.5-kb *SalI-SalI* fragment in pSM5	This study
pSM7	6.6-kb *SmaI-EagI* fragment from pSM1 in YEp24	This study
pSM8	5-kb *SalI-SalI* fragment containing *GLN1* in YEp24	This study

in buffer, and sonicated to disperse clumps. Cells were divided into six 1-ml portions and treated with 0, 10, 15, 20, 25, or 30 µl of ethyl methanesulfonate (EMS). After 70 min at 30°C the cells were washed three times with buffer, suspended in 1 ml of a 5% sodium thiosulfate solution, and incubated for 30 min at 30°C. The cells were washed three times with buffer, suspended in buffer, and plated out to determine levels of survival. Those samples of EMS-treated cells which had survival rates of 5 to 20% were chosen for mutant screening. The strains are shown in Table 1.

Minimal yeast cultures were grown in yeast nitrogen base (Difco Laboratories, Detroit, Mich.) without amino acids and ammonium sulfate but containing 2% glucose as a carbon source. Each nitrogen source was added to a concentration of 0.1% (glutamate, glutamine, aspartate, proline,

alanine, serine) or 0.2% (ammonium sulfate), and amino acid and purine or pyrimidine supplements were added as specified by Sherman et al. (25). Mating, sporulation of diploids, and tetrad analysis were also performed by using the methods of Sherman et al. (25). Transformation of DNA into yeast cells was accomplished by using the lithium acetate method (14).

Enzyme assays. Yeast cells were grown in 25-ml cultures to prepare extracts for enzyme assays. The cultures were shaken at 30°C until they reached mid-log phase, and then the cells were harvested by filtration, washed with water, and frozen at −70°C or used immediately. Extracts were prepared by using the method of Mitchell (Ph.D. thesis), with some modifications. Cells were suspended in extract buffer (0.1 M Tris hydrochloride [pH 7.5], 1 mM phenyl-

methylsulfonyl fluoride, and 1 mM dithiothreitol, with or without 10% glycerol) and vortexed six times for 30 s with glass beads (diameter, 425 to 600 μm). Disrupted cells were centrifuged for 15 min in a microfuge (Brinkmann Instruments Inc., Westbury, N.Y.), and the resulting supernatant was used for enzyme assays. NADP-GDH and NAD-GDH activities were assayed by using the methods of Doherty (4) and Corman and Inamdar (2), respectively, except that both enzyme activities were assayed in 0.1 M Tris hydrochloride buffer at pH 8. Glutamate synthase activity was assayed by using the method of Roon et al. (24), and glutamine synthetase activity was assayed as described previously (20).

Protein purification and generation of antibody. NAD-GDH was purified from a 20-liter, early-stationary-phase glucose-glutamate culture of *ure2-1* strain BC61 grown in a fermentor at 30°C with constant agitation. Cells were suspended in TGED buffer (20% glycerol, 0.1 M Tris hydrochloride [pH 7.5], 1 mM dithiothreitol, 1 mM EDTA) and disrupted with a bead beater cell homogenizer (Biospec Products, Bartlesville, Okla.). Cell debris was pelleted by centrifugation for 60 min at 35,000 rpm, and the resulting supernatant was passed over a Trisacryl M-DEAE column (6 cm by 2.5 cm in diameter). NAD-GDH activity was eluted with a 0 to 0.5 M NaCl gradient in TGED buffer, and the fractions having significant enzyme activity were pooled. Ammonium sulfate was added to a concentration of 40%, and the resulting pellet was suspended in sieve buffer (50 mM Tris hydrochloride [pH 8], 20 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol). The mixture was applied to an A1.5M sieving column (90 by 2.5 cm), and 4-ml fractions were collected. Fractions with significant NAD-GDH activity were pooled and dialyzed for 6 h against 400 volumes of TGED buffer. The dialysate was applied to an AGNAD (agarose-NAD) column (30 by 2.5 cm), the column was washed with TGED buffer and with 10 mM NAD^+ in TGED buffer, and the NAD-GDH activity was eluted with a 0 to 0.3 M NaCl gradient in TGED buffer. Fractions containing NAD-GDH activity were pooled and applied to a MonoQ fast protein liquid chromatography (FPLC) column (5 by 50 mm), washed with TGED buffer, and eluted with a 0 to 1 M NaCl gradient in TGED buffer. NAD-GDH activity eluted in two 1-ml fractions.

The protein in these fractions was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), using a 7.5% gel with a 4% stacker and the conditions described by Laemmli (16). Approximately 80% of the protein that was detectable by Coomassie blue staining of the gel migrated as a 115-kilodalton (kDa) species, and the remainder appeared as faster-migrating bands. On the basis of its emergence as the major band found in the protein purification procedure and on the basis of previous reports of the size of the denatured NAD-GDH subunit (27), we concluded that the 115-kDa band was NAD-GDH. Protein was isolated from this band by electroelution from the excised band for 8 h at 20 mA in Laemmli buffer, using a concentration cup (ISCO, Inc., Lincoln, Nebr.). When the electroeluted protein was analyzed by SDS-PAGE, the predominant band was a 115-kDa band, but a number of lower-molecular-weight bands also appeared; these bands were roughly the same sizes as bands obtained by SDS-PAGE of the purest FPLC fraction of NAD-GDH. This suggests that the 115-kDa protein was unstable to the SDS-PAGE process and that the FPLC fraction was more than 80% pure.

Antibody to NAD-GDH was made by injecting four New Zealand White rabbits with either native protein from the FPLC fraction or the electroeluted 115-kDa protein; two rabbits received native protein, and two received denatured protein. In all, six injections containing 50 μg of protein were made into each rabbit, at 2-week intervals. The first two injections contained protein that was diluted to a volume of 0.5 ml in PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4) and mixed with 0.5 ml of Freund complete adjuvant, and the remaining injections were the same except that they were mixed with Freund incomplete adjuvant instead of Freund complete adjuvant. The rabbits were bled before the first injection and after injections 3, 5, and 6, and the blood was centrifuged to remove cells. The resulting supernatants from these centrifugations were divided into 1-ml samples and stored at −70°C. The specificity of the antibody generated against NAD-GDH was tested by Western blotting (26), with modifications to the original method. Protein from the purest FPLC fraction was subjected to SDS-PAGE and blotted onto a nitrocellulose filter. The filter was blocked with 2% bovine serum albumin in TBS buffer (20 mM Tris hydrochloride [pH 7.5], 0.5 M NaCl) for 2 h and bound with the primary antibody diluted 1:100 in 5% nonfat dry milk in TBS buffer overnight at 4°C. After three washes with TBS buffer, the filter was incubated with secondary antibody, a goat anti-rabbit–horseradish peroxidase conjugate that was diluted 1:1,000 in 5% nonfat dry milk in TBS buffer for 3 h at room temperature. The filter was washed three times with TBS buffer and incubated with a solution containing 3 mg of 1-chloro-2-naphthol per ml and 0.01% H_2O_2 in a 1:5 mixture of methanol and TBS buffer. Antibody from the first two postinjection blood samples from all four rabbits reacted strongly with the 115-kDa band and also reacted with some of the slower-migrating bands, whereas antibody from the preimmune blood samples did not bind the 115-kDa band (data not shown).

λgt11 screening and cloning of GDH2. Antiserum from a rabbit injected with electroeluted protein was incubated with extract from bacterial strain Y1090 to bind antibodies which might react with the host strain used for λgt11 screening (as described by K. Peterson, personal communication). A 5-liter culture of strain Y1090 was pelleted and then suspended in TBS buffer, and the cells were disrupted with a French press. The resulting extract was divided into three portions, and 3 ml of rabbit serum was incubated with one portion for 10 h at 4°C. The debris was pelleted, another portion of extract was added, and the mixture was incubated for 10 h at 4°C. This procedure was repeated once more, and the final supernatant was used for antibody screening. λgt11 expression library screening was performed as described by Huynh et al. (13), except that we used 5% nonfat dry milk as a blocking agent and goat anti-rabbit antibody conjugated to horseradish peroxidase as the secondary antibody. The yeast genomic λgt11 library and bacterial strains Y1090 and Y1089 were kindly supplied by Richard Young.

DNAs were obtained from the phages identified by the antibody (19) and were labeled by using a nick translation kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Poly(A)$^+$ RNA was isolated as described previously (10) from yeast strains BC61 and P6-1a, which make high and low levels, respectively, of NAD-GDH. The RNA was electrophoresed through a formaldehyde gel and transferred to a Gene Screen hybridization transfer membrane as described previously (19), except that the gel was not treated with alkali before the transfer. RNA was fixed to the filters with baking and UV illumination as described by the manufacturer (NEN Research Products, Boston, Mass.), and the

filter was prehybridized for 3 h at 60°C in prehybridization fluid (4× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.5% nonfat dry milk, 1% SDS). The filter was cut into nine strips, each of which contained lanes of RNA isolated from strains BC61 and P6-1a, and the strips were placed into separate bags with prehybridization fluid to which labeled, denatured probe was added. Hybridization continued overnight at 60°C, and the filters were washed three times for 30 min with 1× SSC–0.1% SDS at 50°C and exposed to type SB5 X-ray film (Eastman Kodak Co., Rochester, N.Y.). The filters that were hybridized with λgt11 isolates λgt11-4 and λgt11-14 had strong signals at about 3.0 kilobases in the strain BC61 RNA lanes but not in the strain P6-1a lanes, whereas all lanes hybridized equally with labeled *TUB2* DNA (data not shown).

DNA from phage clone λgt11-14 was labeled by nick translation for use as a hybridization probe to isolate a full-length, YCp50 derivative of *GDH2* from a yeast genomic clone bank (kindly provided by K. Pfeifer). *Escherichia coli* YMC9 harboring the library was probed as described previously (19) to identify colonies that contained plasmids with the *GDH2* gene. The plasmids are shown in Table 1.

DNA manipulations. DNA fragments for cloning were isolated as described previously (1), and Southern analysis was performed as described previously (23).

Chemicals and enzymes. Amino acids, EMS, phenylmethylsulfonyl fluoride, horseradish peroxidase–goat anti-rabbit conjugate, and glass beads were purchased from Sigma Chemical Co., St. Louis, Mo., and Freund adjuvant was obtained from GIBCO Laboratories, Grand Island, N.Y. Restriction endonucleases and the Klenow fragment were purchased from New England BioLabs, Inc., Beverly, Mass., Bethesda Research Laboratories, Inc., Gaithersburg, Md., and International Biotechnologies Inc. NAD⁺, NADH, and NADPH were obtained from Boehringer Mannheim Biochemicals, and column resins for protein purification were purchased from Pharmacia, Inc., Piscataway, N.J. (AGNAD, Trisacyl M-DEAE, and FPLC MonoQ) and from Bio-Rad Laboratories, Richmond, Calif. (A1.5M sieve).

RESULTS

Attempted isolation of mutants that lack NAD-GDH. We attempted to isolate a yeast strain that lacked NAD-GDH on the basis of the assumption that such a strain would not be able to utilize glutamate as a sole nitrogen source. To that end, we mutagenized several yeast strains, including adenine-requiring strain SM10-2a, with EMS and replica plated survivors from glucose-glutamate-ammonia-adenine plates onto glucose-glutamate-adenine plates. In all, 80,000 to 100,000 colonies were screened in several experiments. Promising candidates were grown in liquid cultures and harvested to make cell extracts for enzyme assays. Cells were grown in glucose-glutamate-ammonia-adenine medium to mid-log phase, washed, and shifted for 2 h to glucose-glutamate-adenine medium to derepress NAD-GDH activity. Most of the mutants which we tested were leaky for growth on glutamate, and none of these was deficient in NAD-GDH activity. However, one mutant, mutant SM10-2a-150, had a tight growth phenotype, and when it was assayed, it was found to have only about 20% of the wild-type level of enzyme activity. Glutamine synthetase activity was also assayed and was found to be normal in this strain, so this mutant was different from previously known regulatory mutants that have altered NAD-GDH levels as well as altered glutamine synthetase levels (3, 5, 21).

TABLE 2. Purification of NAD-GDH

Step	Sp act (nmol/min per mg of protein)	Yield (%)
Crude extract	19	100
DEAE	390	79
(NH₄)₂SO₄, sieve	1,700	46
Agarose-NAD	2,800	30
MonoQ FPLC	1,900	17

Mutant SM10-2a-150 was crossed with strain SM14-5d, diploids were sporulated, and the progeny were analyzed. The glutamate growth phenotype segregated 2:2, suggesting that a single mutation was responsible for the phenotype. A segregant from this cross unable to utilize glutamate was crossed again with a wild-type strain, and a number of progeny were scored for growth on glutamate and for NAD-GDH activity. Surprisingly, while two segregants from each tetrad failed to grow on glucose-glutamate-adenine-uracil medium, some of these strains had normal NAD-GDH activity. The NAD-GDH defect also segregated 2:2, but the two phenotypes did not cosegregate. Thus, strain SM10-2a-150 possesses two unlinked mutations, one responsible for the growth defect and the other responsible for the NAD-GDH deficiency. The nature of the former mutation remains unknown, and we named the mutation affecting NAD-GDH *gdh2-1*.

Strain SM10-2a-150 was backcrossed several times with wild-type strains to yield *gdh2-1* strain SM88-2a. We grew strains MB1000 and SM88-2a in glucose-glutamate-uracil medium and made cell extracts. A portion of each extract was kept on ice, and another portion was heated at 55°C for 5 min and then put on ice. When NAD-GDH assays were performed on the four types of extract, we found that the enzyme activity of the wild-type strain had decreased by only 10%, while the enzyme activity of the mutant strain had decreased by 95%. This implied that the *gdh2-1* allele was a mutation in the gene for NAD-GDH which caused the synthesis of a temperature-sensitive enzyme. However, the temperature sensitivity of the enzyme in this strain did not affect the growth of the strain on glutamate or any other nitrogen source tested at any temperature (data not shown).

Cloning of the *GDH2* gene. Because the only *gdh2* strain which we isolated had no known phenotype, we could not use it to clone the *GDH2* gene by complementation. Therefore, we isolated the protein in order to raise antibody to it, so that a clone could be identified from a λgt11 yeast genomic clone bank. The NAD-GDH protein was purified approximately 150-fold (Table 2) as described above to more than 80% homogeneity. On the basis of its elution from an A1.5M molecular sieve, the native enzyme appeared to have a molecular weight of about 400 × 10³, and SDS gel electrophoresis determined that the subunit molecular weight was about 115 × 10³; therefore, the enzyme appeared to be a tetramer. Purification of NAD-GDH has been reported previously by Doherty (4) and by Uno et al. (27), who found that the native enzyme has a molecular weight of 450 × 10³ and a subunit molecular weight of 100 × 10³. Both the purified native enzyme and an SDS gel-purified fraction were injected into rabbits to generate antibody.

Blood serum from a rabbit injected with the gel-purified 115-kDa protein band was used to screen roughly 500,000 λ phage plaques. Nine plaques initially gave a positive signal, and phage from them were plaque purified. In order to identify the candidates that were most likely to harbor
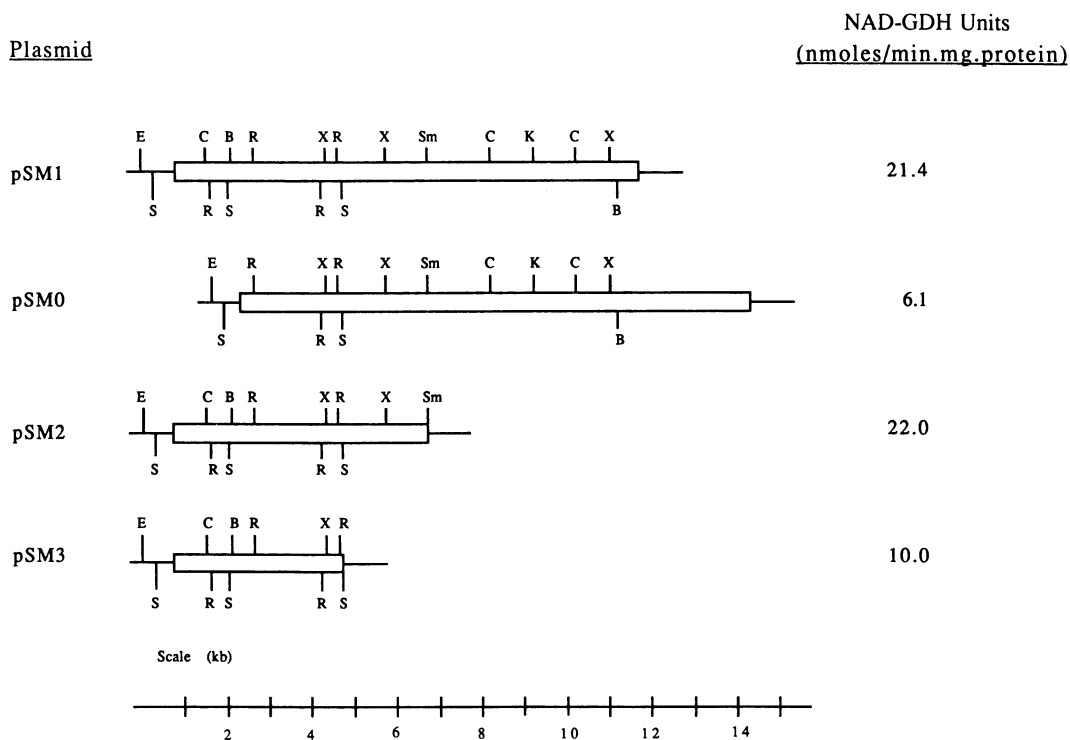
FIG. 1. Complementation of strain SM88-2a with plasmids containing *GDH2* fragments. Plasmids were transformed into strain SM88-2a, triplicate cultures were grown in glucose-glutamate-adenine medium, and NAD-GDH assays were performed on cell extracts. *GDH2* strain SM88-2d was grown in the same medium supplemented with uracil and was found to have 27.5 NAD-GDH units, and strain SM88-2a transformed with plasmid YCp50 had 6.9 NAD-GDH units. The lines indicate YCp50 vector sequences, and the boxes indicate yeast sequences. Abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eag*I; K, *Kpn*I; R, *Eco*RI; S, *Sal*I; Sm, *Sma*I; X, *Xho*I. DNA to the right of the *Bam*HI site in pSM0 was not mapped, and *Eco*RI sites to right of the *Sma*I site in pSM0 or pSM1 were not mapped.

fragments of the *GDH2* gene, labeled DNAs isolated from the phage were hybridized to RNAs isolated from *ure2-1* yeast strain BC61 and from *gln3-1* strain P6-1a; NAD-GDH activity is about 30- to 50-fold greater in *ure2* strains than in *gln3* strains (3). If *GDH2* were transcriptionally regulated, a *GDH2* clone should hybridize more strongly to RNA isolated from strain BC61 than to RNA isolated from strain P6-1a. Two clones, λgt11-4 and λgt11-14, hybridized to a 3.0-kilobase RNA from the *ure2* strain but did not hybridize to RNA from the *gln3* strain (data not shown) and so were analyzed further. Restriction enzyme analysis indicated that the insertions contained in the two phages were overlapping, and DNA from the phage with the larger insertion, λgt11-14, was used to isolate a full-length copy of the *GDH2* gene from a YCp50 yeast genomic clone bank. Phage DNA was labeled and used as a probe in colony hybridization to bacterial colonies harboring YCp50 recombinant plasmids. In an initial screening of 5,000 colonies, 5 candidates were selected for further study; all 5 had common restriction fragments.

To determine which, if any, of the five candidates might contain a functional copy of the *GDH2* gene, the plasmids were transformed into *gdh2-1* yeast strain SM88-2a. Uracil prototrophs were selected for each clone, and they were grown in glucose-glutamate medium along with strain SM88-2a. Extracts were made from the harvested cells and assayed for NAD-GDH activity. Transformants of one of the plasmids, pSM1, had almost as much NAD-GDH activity as the wild-type strain, suggesting that this plasmid contained a functional *GDH2* gene (Fig. 1).

To obtain genetic evidence that plasmid pSM1 encodes the *GDH2* gene, we determined whether yeast sequences on the plasmid were linked to the locus defined by the *gdh2-1* allele. The 2.3-kilobase pair (kb) *Cla*I-*Cla*I fragment (Fig. 1), which contained a *Kpn*I site, was subcloned into the integrating plasmid YIp5. The resulting plasmid, pSM4, was linearized with restriction enzyme *Kpn*I to direct integration of the plasmid into the yeast genome at sequences homologous to the 2.3-kb *Cla*I-*Cla*I fragment. The linearized plasmid was transformed into *gdh2-1* strain SM88-2a. Uracil prototrophs were selected and crossed with *GDH2 ura3-52* strain PM38. Diploids were sporulated, and the progeny were scored for uracil prototrophy and for NAD-GDH activity. In all 10 tetrads analyzed, uracil prototrophy cosegregated with low NAD-GDH activity, and both markers segregated 2:2. This demonstrated linkage of the insertion of plasmid pSM1 to the *gdh2-1* locus, indicating that the plasmid contained the *GDH2* gene.

We next constructed subclones of pSM1 to determine which portion of the 11-kb insertion was necessary for expression of functional NAD-GDH activity. Because clone pSM0 was unable to complement strain SM88-2a for NAD-GDH activity (Fig. 1), it seemed likely that the *GDH2* gene might be contained within the 6.6-kb *Sma*I-*Eag*I fragment of pSM1. Both this fragment and the 4.3-kb *Sal*I-*Sal*I fragment were subcloned back into YCp50 to generate plasmids pSM2 and pSM3, respectively. When transformed into strain SM88-2a, pSM2 complemented for *GDH2* activity as well as pSM1, whereas pSM3 restored only a fraction of the wild-type activity (Fig. 1).

TABLE 3. Effect of the *GDH2* allele on utilization of
various nitrogen sources

Nitrogen source(s)	Growth rate (no. of generations/h) of[a]:		
	Strain SM124-1c (*GDH2*)	Strain SM124-2d (*gdh2Δ2*)	Strain SM88-2a (*gdh2-1*)
Glutamate	0.52	0.09	0.49
Glutamate + adenine	0.49	0.42	0.51
Glutamate + NH$_4^+$	0.59	0.56	0.50
Serine	0.45	0.47	0.40
Alanine	0.39	0.27	0.39

[a] Growth rates were determined in triplicate for minimal glucose cultures supplemented with leucine and the nitrogen sources indicated; glutamate, alanine, and serine were added to concentrations of 0.1%, (NH$_4$)SO$_4$ was added to a concentration of 0.2%, and adenine was added to a concentration of 0.004%. The strain SM88-2a cultures were also supplemented with uracil.

Null *GDH2* allele (*gdh2Δ2::LEU2*). We constructed a deletion-disruption allele of the *GDH2* gene to replace the wild-type gene in the yeast genome. First, plasmid pSM5 was constructed by subcloning the 5.0-kb *SmaI-ClaI* fragment from pSM1 into YIp5. The 2.5-kb *SalI-SalI* fragment was excised from this plasmid and replaced with the 2.2-kb *SalI-XhoI* fragment containing the *LEU2* gene from plasmid YEp24, to yield plasmid pSM6. Nuclease S1 analysis indicated that the transcriptional start site for *GDH2* lies within the 2.5-kb *SalI-SalI* fragment, about 150 base pairs downstream from the rightmost *SalI* site as the gene is shown in Fig. 1, so that the portion of the gene that encodes the amino-terminal five-sixths of the protein has been removed (data not shown). Plasmid pSM6 was digested with *ClaI* (partially) and with *XhoI* to direct homologous recombination of the null allele of *GDH2* (marked by the *LEU2* gene) into the yeast genome. The *ClaI-XhoI* fragment was used to transform diploid strain P24-000 to leucine prototrophy, and the transformants were sporulated. DNAs were isolated from parent strain P24-000, from *LEU2* transformants of this strain, and from *LEU2* and *leu2* haploids from the sporulated diploids. These DNAs were digested with *ClaI* and hybridized in a Southern blot with the 1.1-kb *EcoRI-EcoRI* fragment from pSM2. The pattern of hybridizing fragments indicated that the null allele had integrated into the chromosome in place of the wild-type allele, as expected (data not shown). Haploid progeny from the diploid were scored for growth phenotypes. In each case all four spores germinated on rich-medium YPD plates, indicating that *GDH2* is not an essential gene. Leucine prototrophy segregated 2:2, and all of the progeny were able to utilize glutamate as a nitrogen source when they were scored on glucose-glutamate medium supplemented with uracil, leucine, histidine, and adenine. When they were assayed for NAD-GDH activity, all of the *LEU2* progeny were deficient, and all of the *leu2* progeny had the wild-type level of the enzyme. The NAD-GDH activity in the null strains was about 5% of the NAD-GDH activity in the wild-type strain (data not shown).

Surprisingly, we found that when they were grown without adenine, *gdh2Δ2::LEU2* strains were significantly impaired in their utilization of glutamate. Strains SM124-1c (*GDH2*) and SM124-2d (*gdh2Δ2::LEU2*) were grown in glucose-glutamate-leucine medium with or without ammonia or adenine, and growth rates were determined (Table 3). Without supplements the *gdh2Δ2::LEU2* strain barely grew, while ammonia and adenine restored wild-type growth. We also found that glucose-glutamate-leucine medium supple-

mented with hypoxanthine or guanine supported near-normal growth of strain SM124-2d, that cytosine and histidine enhanced growth somewhat less, and that uracil and xanthine did not enhance growth at all (data not shown). Hypoxanthine does not have an amino group as adenine and guanine do, but it can be converted to either nucleotide. This suggests that ammonia is not provided by the deamination of adenine and guanine. Also, since xanthine, which cannot be converted to adenine or hypoxanthine, did not enhance growth, it is unlikely that the purine ring can be catabolized to yield ammonia. Instead, as glutamine is required for the biosynthesis of nucleotides and of some amino acids, including histidine, these compounds probably improve growth by sparing glutamine.

In contrast to strain SM124-2d (*gdh2Δ2::LEU2*), strain SM88-2a, which carried the temperature-sensitive mutation *gdh2-1*, grew well in glucose-glutamate medium in the absence of adenine (Table 3). This ability apparently resulted from the higher NAD-GDH level (5.2 U/mg of protein) in strain SM88-2a compared with the NAD-GDH level in strain SM124-2d (0.8 U/mg of protein). Wild-type strain SM124-1c and both mutant strains grew readily in media containing serine or alanine as a sole source of nitrogen (Table 3), indicating that *S. cerevisiae* can obtain ammonia from these amino acids by pathways that do not involve glutamate as an intermediate. On the other hand, strain SM124-2d hardly grew with aspartate or proline as a source of nitrogen (data not shown), which is in accord with the view that glutamate is an intermediate in the deamination of these amino acids.

Role of NAD-GDH in ammonia assimilation. The major pathway for ammonia assimilation is the reductive amination of α-ketoglutarate by ammonia and NADPH, which is catalyzed by the enzyme NADP-GDH, the product of the *GDH1* gene (9). However, mutants which lack this enzyme have not completely lost their ability to use ammonia as a source of nitrogen. They grow in medium with ammonia as a sole source of nitrogen one-half as fast as the wild-type strain, and addition of glutamate fully restores the original growth rate (9). We considered the possibility that the residual ability of mutants which lack NADP-GDH to utilize ammonia was due entirely or partially to the ability of NAD-GDH to catalyze the reductive amination of α-ketoglutarate by ammonia and NADH. To explore this possibility, we attempted to isolate mutants of strain BC34 (*gdh1-6*) that had lost the ability to use ammonia as a source of nitrogen. We mutagenized this strain with EMS and replica plated 15,000 colonies from glucose-glutamate-ammonia-adenine plates onto glucose-ammonia-adenine plates. We found 11 mutants which grew well on glucose-glutamate-ammonia-adenine plates, but poorly or not at all on glucose-ammonia-adenine plates. These strains were grown in glucose-glutamate-ammonia-adenine cultures to mid-log phase and were shifted to glucose-ammonia-adenine and glucose-glutamate-adenine media for 2 h. The cells were harvested, extracts were prepared, and NAD-GDH and glutamate synthase activities were measured in the extracts from the glutamate- and ammonia-grown cultures, respectively. None of the mutants was defective for NAD-GDH (data not shown), but two mutants, mutants BC34-9 and BC34-10, were deficient in glutamate synthase, the enzyme which is responsible for the reductive amination of α-ketoglutarate by glutamine and NADH.

To establish that the inability of mutants BC34-9 and BC34-10 to use ammonia as a source of nitrogen depended both on the *gdh1-6* allele and on a new mutation which eliminated glutamate synthase activity, we crossed the mu-

TABLE 4. Enzyme activities and growth rates of the wild-type strain and of strains with mutations in *GDH1* or *GLT1* or both

Strain	Relevant genotype	Sp act (nmol/min per mg of protein)[a]		Growth rate (no. of generations/h) in[b]:	
		NADP-GDH	Glutamate synthase	Glucose-glutamate-ammonia medium	Glucose-ammonia medium
SM50-1a	*GDH1 GLT1*	200	6.5	0.44	0.49
SM50-1b	*gdh1 GLT1*	<1	7.0	0.44	0.23
SM50-1c	*GDH1 glt1-2*	190	0.7	0.43	0.49
SM50-2c	*gdh1 glt1-2*	<1	<0.1	0.38	0.07
SM77-2c	*GDH1 GLT1*	210	4.4	0.51	0.49
SM77-2b	*gdh1 GLT1*	<1	8.4	0.46	0.25
SM77-3b	*GDH1 glt1-1*	200	0.1	0.50	0.49
SM77-3c	*gdh1 glt1-1*	<1	<0.1	0.46	0.05

[a] Duplicate glucose-glutamate-ammonia-adenine-uracil cultures were grown to mid-log phase, harvested, washed twice with water, and suspended in glucose-ammonia-adenine-uracil medium. The cells were incubated for 2 h and then harvested to make extracts.

[b] To determine growth curves, cultures were grown in triplicate in media supplemented with 0.002% uracil and 0.004% adenine.

tants to wild-type strain MB1000 and examined the progeny. In all, we analyzed 20 tetrads, 10 from each cross. Glutamate synthase activity segregated 2:2 in eight tetrads from the cross BC34-9 × MB1000 and 3:1 (wild type-mutant) in the other two tetrads. In the cross BC34-10 × MB1000, glutamate synthase activity segregated 2:2 in eight tetrads, 3:1 (wild type-mutant) in one tetrad, and 1:3 (wild type-mutant) in the remaining tetrad. The *gdh1-6* and *ade1-100* alleles segregated 2:2 in all 20 tetrads. The existence of two loosely linked genes that determine glutamate synthase activity has been reported previously (8). Whether our segregation pattern resulted from multiple glutamate synthase genes or from a phenomenon such as gene conversion is unclear. Nonetheless, of the 80 segregants that were analyzed from the 20 tetrads, those which lacked both NADP-GDH activity and glutamate synthase activity failed to grow with ammonia as a nitrogen source, whereas the segregants with either activity were able to grow (data not shown).

A segregant of the cross BC34-9 × MB1000 that was unable to grow on ammonia was crossed with a segregant having the same growth phenotype but the opposite mating type from the cross BC34-10 × MB1000. We found that diploids, which were isolated on the basis of zygote morphology and were tested for the ability to sporulate, did not grow on glucose-ammonia-adenine plates, indicating that the mutations were in the same gene. For simplicity, we attributed the deficiency in glutamate synthase activity of these strains to single mutations, and the locus defined by these mutations was designated *GLT1*. The allele that was isolated from strain BC34-10 was designated *glt1-1*, and the allele

that was isolated from strain BC34-9 was designated *glt1-2*. These mutations are probably allelic to *gus* and *ama* (8, 9).

Next, we attempted to determine the effects of the *glt1* mutation in cells with functional NADP-GDH activity. To that end, strains BC34-9 and BC34-10 were each backcrossed three times to *GDH1 GLT1* derivatives of strain MB1000. The growth rates of the *GDH1 GLT1*, *GDH1 glt1*, *gdh1 GLT1*, and *gdh1 glt1* progeny of the final cross were measured (Table 4), along with the NADP-GDH and glutamate synthase activities of the eight strains. While a loss of NADP-GDH activity resulted in an approximately twofold reduction in the growth rate on ammonia, the strains that lacked only glutamate synthase activity grew as well as the wild-type strains. In contrast, the *gdh1 glt1* double mutants grew hardly, if at all, on ammonia. We also tested the growth of the eight strains with limiting amounts of ammonia, but the *GDH1 glt1* strains could not be distinguished from the *GDH1 GLT1* strains (data not shown). The *gdh1 GLT1* strains grew a little more slowly than these strains, and as expected, the double mutants grew very poorly.

The results described above do not exclude the possibility that NAD-GDH participates in glutamate synthesis in ammonia-grown cells. Potentially, both NAD-GDH and glutamate synthase could be required for glutamate synthesis in the absence of NADP-GDH, and the loss of either could prevent growth on ammonia. To test this possibility, we deleted the *GDH2* gene from *gdh1-6* strain PM35; the disruption was carried out as described above for disruption of *GDH2* in strain P24-000. As shown in Table 5, strain SM1 (*gdh1-6 gdh2Δ2::LEU2*) grew with ammonia as a source of

TABLE 5. Ammonia assimilation by NAD-GDH and glutamine synthetase

Strain	Relevant genotype[a]	Sp act (nmol/min per mg)[b]		Growth rate (no. of generations/h) in[c]:	
		NAD-GDH	Glutamine synthetase	Glucose-ammonia medium	Glucose-glutamate-ammonia medium
SM2	*gdh1*	2.8	540	0.31	0.45
SM1	*gdh1 gdh2*	1.8	520	0.33	0.55
SM3	*gdh1 glt1*	2.2	ND[d]	NG[e]	0.38
SM4	*gdh1 glt1 (GDH2 [2 μ])*	195	ND	0.28	0.41
SM6	*gdh1 gln3*	1.9	100	NG	0.20
SM5	*gdh1 gln3 (GLN1 [2 μ])*	5.6	2,900	0.10	0.29

[a] 2 μ indicates that the gene was carried on a multicopy plasmid.

[b] Enzyme assays were performed by using duplicate extracts prepared from glucose-ammonia cultures supplemented with 0.004% adenine (strains SM1, SM2, SM5, and SM6), with 0.002% histidine (strain SM4), or with 0.002% histidine and 0.002% uracil (strain SM3); strains SM3, SM4, SM5, and SM6 were grown to mid-log phase in glucose-glutamate-ammonia medium and shifted to glucose-ammonia medium for 2 h before the cells were harvested.

[c] Strains were grown in triplicate in 25-ml minimal glucose-ammonia or glucose-glutamate-ammonia cultures supplemented as described in footnote b.

[d] ND, Not done.

[e] NG, No growth.

nitrogen as well as strain SM2 (*gdh1-6 GDH2*); thus, the lack of NAD-GDH did not exacerbate the deficiency in ammonia assimilation that resulted from the loss of NADP-GDH. The lack of any contribution of NAD-GDH to ammonia assimilation was also reflected in the fact that the level of NAD-GDH activity in cells of strain SM2 grown with ammonia as a source of nitrogen (Table 5) was as low as the level in corresponding cells of the wild type, compared with the high levels (20 to 30 nmol/min per mg of protein) found in cells of either strain grown with glutamate as a source of nitrogen. Furthermore, incubation of strain SM3 (*gdh1-6 glt1-1*) in a medium containing ammonia but lacking glutamate did not result in an increase in the level of NAD-GDH (Table 5).

In contrast to NAD-GDH, glutamine synthetase appears to play a role in the assimilation of ammonia. The levels of this enzyme in ammonia-grown cells of strains SM2 and SM1 (Table 5) and of the wild type (data not shown) were high when they were compared with the low levels of this enzyme in cells grown with glutamine as a source of nitrogen. Therefore, it is likely that glutamine synthetase is responsible for the ability of strain SM1 cells to utilize ammonia as a source of nitrogen. This hypothesis is supported by the observation that a strain carrying in addition to the *gdh1-6* mutation the mutation *gln3Δ4::LEU2*, which prevents the increase in the level of glutamine synthetase in cells grown in the absence of glutamine, is unable to utilize ammonia as a source of nitrogen (W. Courchesne, personal communication) (Table 5). This inability can be overcome by introducing a high-copy-number plasmid carrying a functional *GLN1* gene, resulting in an increase in glutamine synthetase activity to a level that is six-fold higher than the level found in cells with a functional *GLN3* gene (compare strains SM5 and SM2 in Table 5). This overproduction of glutamine synthetase could result in a glutamate deficiency in cells that are already deficient in the ability to utilize ammonia for the synthesis of glutamate and may be responsible for the slow growth of strain SM5 on ammonia as a source of nitrogen.

Although the results described above exclude NAD-GDH from any physiological role in ammonia assimilation, we found that the overproduction of NAD-GDH which resulted from the introduction of a multicopy plasmid carrying a functional *GDH2* gene into cells of strain SM3 (*gdh1-6 glt1-1*) enabled the resulting strain, strain SM4, to utilize ammonia as a source of nitrogen (Table 5). Apparently, when NAD-GDH is present in very high intracellular concentrations, it can catalyze the reductive amination of α-ketoglutarate by ammonia and NADH.

DISCUSSION

All cellular nitrogen is incorporated into macromolecules by way of the amino group of glutamate and the amide group of glutamine. Glutamate provides approximately 85% of the nitrogen, which is used for the synthesis of amino acids, while glutamine provides the remaining 15%, which is used for the synthesis of purines, pyrimidines, amino sugars, histidine, tryptophan, asparagine, NAD^+, and p-aminobenzoate (29). Therefore, cells growing with glutamate as a source of nitrogen are directly provided with 85% of their nitrogen requirement, but must generate ammonia from glutamate to be used for the synthesis of glutamine by the enzyme glutamine synthetase.

Our results show that NAD-GDH has the major, but not exclusive, responsibility for generating ammonia from glutamate. Mutants with a disruption in *GDH2*, the structural gene for this enzyme, grow at one-fifth the rate of a strain

with the wild-type gene (Table 3). On the other hand, a strain with a temperature-sensitive NAD-GDH that reduces the functional enzyme activity to approximately one-fifth of the level found in cells of the wild-type strain is not affected in its ability to use glutamate as a source of nitrogen (Tables 2 and 3). Apparently, the enzyme is normally produced at a level that is greatly in excess of the level necessary for providing cells growing on glutamate with sufficient ammonia for the synthesis of glutamine.

We found that NAD-GDH encoded by the *GDH2* gene is not solely responsible for the generation of ammonia from glutamate. Cells with a disruption of *GDH2* grow as well as the wild type in media with glutamate as the major nitrogen source when these media are supplemented with adenine (Table 3). Apparently, provision of adenine as a source of purine nucleotides obviates the need for a glutamine-requiring pathway and thus allows minor mechanisms of ammonia generation from glutamate to satisfy the residual need for glutamine. We obtained mutants of the *gdh2Δ1::LEU2* strain that were completely unable to grow with glutamate as a nitrogen source in adenine-deficient medium (unpublished data). These double mutants also failed to grow with aspartate or proline as the major source of nitrogen, but retained the ability of the wild-type strain to grow with alanine or serine as a sole source of nitrogen. Apparently, the organisms can generate ammonia from these amino acids by a pathway that does not involve glutamate as an intermediate. An L-serine (L-threonine) deaminase that enables yeast cells to use these amino acids as sources of nitrogen has been described previously (22). It is possible that the residual ability of the mutants which lack NAD-GDH to generate ammonia from glutamate depends on the conversion of glutamate to these amino acids and that the double mutants are deficient in this ability.

In agreement with previous reports (8, 28), we found, that the ability of mutants with mutations in *GDH1*, the structural gene for NADP-GDH, to grow with ammonia as a sole source of nitrogen (although they grow more slowly than the wild-type strain) depends on glutamate synthase. Mutants defective both in this enzyme and in NADP-GDH fail to utilize ammonia as a sole source of nitrogen. The ability to utilize the glutamate synthase pathway for the synthesis of glutamate from ammonia depends on the intracellular level of glutamine synthetase, the product of *GLN1*. This enzyme provides the glutamine which is subsequently used by glutamate synthase together with α-ketoglutarate and NADH for the synthesis of glutamate. It is for this reason that a mutant defective in *GDH1* as well as *GLN3*, whose product is required for raising the intracellular level of glutamine synthetase in response to glutamine deficiency (21), fails to utilize ammonia as a sole source of nitrogen; introduction of a multicopy plasmid carrying *GLN1* results in increased production of glutamine synthetase in the double mutant and allows it to use ammonia as a sole source of nitrogen (Table 5).

We found that NAD-GDH normally plays no role in ammonia assimilation; a strain which lacks both NADP-GDH (*gdh1*) and NAD-GDH (*gdh2*) grows as well with ammonia as a source of nitrogen as a strain which lacks only NADP-GDH. Nevertheless, a greatly increased intracellular concentration of NAD-GDH enables a mutant which lacks both NADP-GDH and glutamate synthase to grow with ammonia as the only source of nitrogen (Table 5). Apparently, in this case the high level of the enzyme makes it possible to generate glutamate from α-ketoglutarate, ammonia, and NADH. The same explanation may be offered for

the reported observation that a mutation in the *URE2* gene that results in an increase in the intracellular level of NAD-GDH in cells grown with ammonia as a source of nitrogen increases the growth rate of a mutant which lacks NADP-GDH in such a medium (9). However, the mutation in *URE2* also results in an increase in the level of glutamine synthetase (17), and it may be that the increased production of this enzyme enhances the pathway which leads from ammonia to glutamate that depends on glutamate synthase.

ACKNOWLEDGMENTS

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