Plant Physiol. (1984) 76, 1009–1013
0032-0889/84/76/1009/05/$01.00/0

Characteristics of Glutamate Dehydrogenase in Mitochondria Prepared from Corn Shoots[1]

Received for publication March 31, 1984 and in revised form August 16, 1984

TOMOYUKI YAMAYA, ANN OAKS*, AND HIDEAKI MATSUMOTO
Institute for Agricultural and Biological Sciences, Okayama University, Chuo 2-20-1, Kurashiki, Okayama 710, Japan (T.Y., H.M.); *and Department of Biology, McMaster University, Hamilton, Ontario, L8S 4K1 Canada* (A.O.)

ABSTRACT

The amination of α-ketoglutarate (α-KG) by NADH-glutamate dehydrogenase (GDH) obtained from Sephadex G-75 treated crude extracts from shoots of 5-day-old seedlings was stimulated by the addition of Ca^{2+}. The NADH-GDH purified 161-fold with ammonium sulfate, DEAE-Toyopearl, and Sephadex G-200 was also activated by Ca^{2+} in the presence of 160 micromolar NADH. However, with 10 micromolar NADH, Ca^{2+} had no effect on the NADH-GDH activity. The deamination reaction (NAD-GDH) was not influenced by the addition of Ca^{2+}.

About 25% of the NADH-GDH activity was solubilized from purified mitochondria after a simple osmotic shock treatment, whereas the remaining 75% of the activity was associated with the mitochondrial membrane fraction. When the lysed mitochondria, mitochondrial matrix, or mitochondrial membrane fraction was used as the source of NADH-GDH, Ca^{2+} had little effect on its activity. The mitochondrial fraction contained about 155 nanomoles Ca per milligram of mitochondrial protein, suggesting that the NADH-GDH in the mitochondria is already in an activated form with regard Ca^{2+}. In a simulated *in vitro* system using concentrations of 6.4 millimolar NAD, 0.21 millimolar NADH, 5 millimolar α-KG, and 5 millimolar glutamate thought to occur in the mitochondria, together with 1 millimolar Ca^{2+}, 10 and 50 millimolar NH_4^+, and purified enzyme, the equilibrium of GDH was in the direction of glutamate formation.

NADH-GDH[2] is easily solubilized from mitochondria from roots (30), shoots (4, 25), and seeds (28) of various plants. Although it is the only mitochondrial enzyme with the potential for the assimilation of NH_4^+, it has been suggested by Miflin and Lea (21) that the NH_4^+ released by the conversion of glycine to serine in the mitochondria (3) is reassimilated via GS in the cytosol. They have also suggested that the principal function of mitochondrial GDH is the oxidation of glutamate (21). However, a part of intramitochondrially generated NH_4^+ from glycine is incorporated into glutamate by mitochondria isolated from pea shoots (11). Chou and Splittstoesser (7), Joy (14), and recently Furuhashi and Takahashi (10) have shown that NADH-GDH (amination reaction), but not NAD-GDH (deamination reaction), is activated by Ca^{2+} *in vitro*. According to the work by Bowman *et al.* (4), NAD is the only detectable pyridine nucleotide in mung bean mitochondria. Thus, concentrations of Ca^{2+} and NADH in the mitochondria could be important in the regulation of NADH-GDH.

The purpose of the present study was to characterize the activation of NADH-GDH by Ca^{2+} and to estimate concentrations of Ca^{2+} and NADH for simulation of the GDH reaction in mitochondria isolated from corn shoots.

MATERIALS AND METHODS

Plant Materials. Corn seeds (*Zea mays* L. var W64A × W182E) were planted in vermiculite and seedlings were grown in a Toshiba Cold Chain growth chamber, model TGS-13L, with a 12-h photoperiod for 5 d. Light (about 490 $\mu E/m^2 \cdot s$) was supplied by Toshiba Yoko lamps DA400 (metal halide lamp) and fluorescent lamps, and the temperature was maintained at 27°C. The plants were watered every morning with a nutrient medium containing 1 mM Ca^{2+} as described previously (18).

Isolation of Mitochondria by Density Gradient Centrifugation. All reagents and buffer solutions were prepared with double glass-distilled H_2O to minimize Ca^{2+} contamination, and all operations were carried out at 4°C. Whole shoots of 5-d seedlings were harvested, washed in water, blotted dry with filter paper, and weighed. The shoots (40 to 60 g in fresh weight) were chopped with razor blades in extraction buffer consisting of 0.4 M mannitol, 0.1 M Hepes-KOH buffer (pH 7.5), 1 mM EDTA, 0.1% (w/v) BSA, and 0.6% (w/v) insoluble PVP, at a ratio of 1 g fresh weight per 3 ml of buffer. The chopped materials were ground with motar and pestle.

The mitochondrial fraction was isolated by modification of the method described by Nishimura *et al.* (26) as described previously by us (32). The purified mitochondria after Percoll discontinuous density gradient were able to oxidize both malate and succinate with a high P/O and RCR ratios and appear to have about 92% of the outer membrane intact (32). Our preparations were almost free of Chl (chloroplast) or catalase (microbody) contamination. These are criteria used by Nishimura *et al.* for assessing mitochondrial purity (26).

Preparation of Submitochondrial Fractions. The purified mitochondrial pellet was treated hypotonically using 1.5 ml of 20 mM Hepes-KOH buffer (pH 7.5). The suspension was mixed with a Teflon-glass homogenizer and incubated in an ice bath for 30 min. The suspension (1 ml) was then centrifuged in the ultracentrifuge equipped with an SW 50-1 rotor at 150,000 *g* for 1 h. The pellet, resuspended in 1 ml of the same buffer, was designated the mitochondrial membrane fraction. The supernatant remaining above the pellet consisted of the soluble matrix

[1] Supported in part by Grants-in-Aid for Science Research from the Ministry of Education, Science and Culture of Japan (No. 57760052 and No. 58390012) and from the National Research Council of Canada (No. A2818).

[2] Abbreviations: GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; α-KG, α-ketoglutarate; 2-ME, 2-mercaptoethanol; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; PES, phenazine ethosulfate.

of the mitochondria.

Purification of NADH-GDH from the Crude Mitochondrial Fraction. The crude mitochondrial pellet prepared from 179 g fresh weight of the corn shoots was suspended in about 90 ml of 0.1 M Tris-HCl buffer (pH 8.5) containing 0.1 mM EGTA and 1 mM 2-ME. The suspension was frozen and thawed twice, placed in a salt-ice mixture, and then sonicated with a Branson sonifier, model 200, operating at power setting 5 on a 50% duty cycle for 2 min. Following the disruption treatment, the suspension was centrifuged at 20,000*g* for 30 min and the supernatant fraction was designated the crude mitochondrial extract. The extract was subjected to 30 to 60% $(NH_4)_2SO_4$ fractionation. The resulting pellet was dissolved in 0.1 M Tris-HCl buffer (pH 8.5) containing 0.1 mM EGTA and 1 mM 2-ME and then dialyzed against the same buffer. The dialyzed preparation was centrifuged and the supernatant fraction was loaded onto a column (1.6 × 10 cm) of DEAE-Toyopearl, equilibated with 50 mM Tris-HCl buffer (pH 8.5) containing 5 mM α-KG, 1 mM 2-ME, and 0.1 mM EGTA. The column was washed with the same buffer and the NADH-GDH was eluted with a linear gradient of KCl (0 to 0.5 M). The NADH-GDH activity was recovered in the portion of the effluent containing approximately 0.12 M KCl. Proteins were then sedimented with 70% $(NH_4)_2SO_4$ and the enzyme was further purified by gel filtration chromatography on Sephadex G-200 column (1.6 × 90 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.5) containing 0.1 M KCl and 1 mM 2-ME. The NADH-GDH activity was observed as a single peak near the void volume of the column, and was free of contaminating EGTA.

When NADH-GDH activity was assayed without purification, the procedures of enzyme extraction from the corn shoots and desalting with Sephadex G-75 were the same as those described by Oaks *et al.* (27).

Enzyme Assays and Analytical Methods. Enzyme activities were assayed spectrophotometrically in a double-beam spectrophotometer (Shimadzu, model UV-150-02) at 30°C. NADH-GDH was assayed routinely in 0.2 M Tris-HCl buffer (pH 8.0), containing 0.266 M $(NH_4)_2SO_4$, 0.16 mM NADH, and 5 mM α-KG with or without 1 mM $CaCl_2$ as described previously (27). However, in critical experiments $(NH_4)_2SO_4$ was used in a concentration range of 2 to 100 mM. NAD-GDH was measured by a modification of the method described by Furuhashi and Takahashi (10). The assay system contained 0.1 M Tris-HCl buffer (pH 8.5), 3 mM NAD, and 50 mM glutamate with or without 1 mM $CaCl_2$. Succinate:Cyt *c* reductase activity was assayed by following reduction of Cyt *c* as described by Douce *et al.* (9). Malate dehydrogenase was assayed by following the oxidation of NADH as described by Priestley and Bruinsma (28). Fumarase was assayed by coupling the reaction with malic enzyme and following the reduction of NADP as described by Hatch (12).

The Ca content in the isolated mitochondrial fraction was determined with an atomic absorption/flame emission spectrophotometer (Shimadzu model AA646, equipped with a Graphite furnace atomizer, model GFA-3). Protein content was determined by the method of Lowry *et al.* (16).

Extraction and Determination of Pyridine Nucleotides. Pyridine nucleotide content was determined by the cycling assay method described by Matsumura and Miyachi (19). During extraction and isolation of mitochondria, 10 μM rotenone and 5 μM antimycin A were included in all the buffer solutions and in the Percoll gradient solutions. The final mitochondrial pellet after the Percoll density gradient was dissolved in 1.1 ml of 0.1 M Bicine-NaOH buffer (pH 8.0). The mitochondrial suspension (0.5 ml) was then transferred to preheated NaOH or HCl solutions which had a final concentration of 0.1 N. After an incubation at 100°C for 2 min, the suspension was cooled and centrifuged. NADH is specifically extracted with an alkaline treatment, while NAD is extracted with an acid treatment (19). Each extract was neutralized by adding an appropriate amount of NaOH or HCl. The extract (50 to 200 μl) was then incubated in brown test tubes in a reaction system containing 0.1 M Bicine-NaOH buffer (pH 8.0), 4 mM EDTA, 0.42 mM MTT, 1.676 mM PES, and 0.5 M ethanol for 5 min at 37°C. The total reaction volume was 1.0 ml. The reaction was started by adding 10 units of alcohol dehydrogenase and was allowed to run for 30 min at 37°C. The reaction was terminated by adding 12 mM iodoacetate and the absorbance was measured at 570 nm. Amounts of NAD(H) were calculated from the standard curves with authentic NAD(H). A blank test without sample or NAD(H) was also carried out.

Source of Chemicals. Bicine, Hepes, Mes, Tris, sucrose, mannitol, PES, Na-glutamate, and rotenone were all purchased from Nakarai Chemicals, Ltd., Japan and were of a specially prepared reagent grade. Fatty acid-free BSA, horse heart Cyt *c* (type III), yeast alcohol dehydrogenase free of NAD(H), chicken liver malic enzyme, and MTT were from Sigma; antimycin A, from Boehringer; Percoll and Sephadex, from Pharmacia. NAD, NADH, and NADP were purchased from Kojin Co. Ltd, Japan and DEAE-Toyopearl was from Toyo Soda MFG Co. Ltd, Japan. All other chemicals were of the highest quality available.

RESULTS

Localization of NADH-GDH in Mitochondria Isolated from Corn Shoots. NADH-GDH is relatively easily solubilized from plant mitochondria and hence the enzyme is thought to be localized in the soluble matrix of mitochondria (4, 28). However, the method of disrupting the mitochondria in those reports (4, 28) involved a relatively vigorous treatment, such as freezing and thawing followed by sonication. To demonstrate the distribution of NADH-GDH in corn mitochondria, we treated mitochondria, purified by centrifugation through a Percoll density gradient, with osmotic shock, a relatively gentle disruption method. The mitochondrial matrix and membrane fractions were then separated by centrifugation (Table I). Most of the fumarase activity (a matrix marker) and succinate:Cyt *c* reductase (a membrane marker) were located in the expected fractions (29). NADH-GDH and malate dehydrogenase, which were thought to be in the mitochondrial matrix (4, 28) were, however, found primarily in the membrane fraction (75%). When the mitochondria were disrupted by harsher treatments (freezing and thawing followed by sonication), about 85% of the NADH-GDH activity was

Table I. *Localization of NADH-GDH in Mitochondria Isolated from Corn Shoots*

The purified mitochondrial pellet was shocked hypotonically using 1.5 ml of 20 mM Hepes-KOH buffer (pH 7.5), suspended with a Teflon-glass homogenizer, and incubated in an ice bath for 30 min. The lysed mitochondrial suspension (1 ml) was centrifuged at 150,000 *g* for 1 h. The resulting pellet was resuspended in 1 ml of the same buffer (mitochondrial membrane fraction). The supernatant fraction remaining above the pellet was designated the mitochondrial matrix fraction.

Fraction	NADH-GDH	Malate Dehydrogenase	Fumarase	Succinate:Cyt *c* Reductase
	μmol substrate utilized·min⁻¹·mg⁻¹ protein			
Lysed mitochondrial fraction	0.476	2.03	0.024	0.116
	% of activity assayed with the lysed mitochondria			
Mitochondrial matrix	25.5	22.6	70.4	1.3
Mitochondrial membrane	77.8	74.8	7.4	89.2

Table II. *Activation of NADH-GDH by Ca^{2+}*

The amination reaction of GDH was measured using a crude preparation desalted on Sephadex G-75 column (27), a 161-fold purified fraction, a lysed mitochondrial fraction, a mitochondrial matrix fraction, and a mitochondrial membrane fraction from shoots of 5-d-old corn. The NADH-GDH activity was measured in the presence or absence of 1 mM $CaCl_2$ in the standard assay system containing 0.2 M Tris-HCl buffer (pH 8.0), 0.266 M $(NH_4)_2SO_4$, 0.16 mM NADH, and 5 mM α-KG.

Treatment	Desalted Crude Fraction	Source of NADH-GDH			
		Purified enzyme	Mitochondrial fractions		
			Lysed	Matrix	Membrane
	μmol NADH oxidized·min^{-1}·mg^{-1} protein				
No addition	0.01	1.40	0.68	0.20	0.65
Ca^{2+} (1 mM)	0.05	10.70	0.73	0.21	0.73

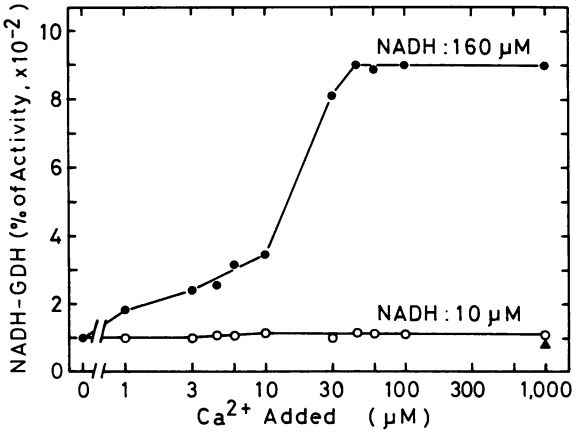

FIG. 1. Effect of Ca^{2+} on the amination reaction of GDH purified from corn shoots. The assay mixture contained 10 (○) or 160 (●) μM NADH, 5 mM α-KG, 100 mM $(NH_4)_2SO_4$, and 1.2 μg of the 161-fold purified enzyme in 0.2 M Tris-HCl buffer (pH 8.0). The NADH-GDH activity was 3.38 and 1.69 nmol NADH oxidized·min^{-1} with 10 and 160 μM NADH, respectively, in the absence of Ca^{2+}. Effect of 2 mM EGTA was also tested in the presence of 160 μM NADH and 1 mM Ca^{2+} (▲).

solubilized, whereas most of the succinate:Cyt *c* reductase was still associated in the membrane fraction (80 to 88%). These results indicated that both NADH-GDH and malate dehydrogenase are loosely associated with the mitochondrial membrane.

Activation by Ca^{2+} of Reductive Amination Reaction of GDH from Corn Shoots. NADH-GDH was prepared in five different ways from 5-d-old corn shoots: (a) a crude extract desalted by passage of Sephadex G-75, (b) a highly purified enzyme fraction, (c) a lysed mitochondrial fraction, (d) a mitochondrial matrix fraction, and finally (e) a mitochondrial membrane fraction. Activities in the presence of 1 mM Ca^{2+} in crude mitochondrial preparations and purified enzyme were 0.10 and 16.1 μmol NADH oxidized/min·mg protein. Thus, a 161-fold purification was achieved, 77% of the original activity was recovered, and all of the contaminating NADH oxidizing activity was removed (data not shown).

The effect of addition of 1 mM Ca^{2+} on the NADH-GDH activity was tested with each of these preparations (Table II). The NADH-GDH in the Sephadex G-75 treated fraction or the highly purified preparation was activated 5.1- and 9.1-fold, respectively, by the addition of Ca^{2+}. With the GDH obtained from the lysed mitochondria, the mitochondrial matrix, or the membrane fraction, Ca^{2+} additions had only a minor effect.

The effect of Ca^{2+} on the NADH-GDH activity was examined further with the highly purified preparation. The response of Ca^{2+} on the amination reaction was tested using fixed concentrations of 200 mM NH_4^+, 5 mM α-KG, and 10 or 160 μM NADH. In the presence of 160 μM NADH, the activation curve was sigmoidal with significant activation seen with 1 μM Ca^{2+} and maximal activation with 45 μM Ca^{2+} (Fig. 1). The NADH-GDH was thus activated about 9-fold by Ca^{2+}. Higher concentrations of Ca^{2+}, up to 10 mM, maintained the activity at the saturated level (results not shown). Addition of 2 mM EGTA completely reversed the activation of the enzyme by 1 mM Ca^{2+}. When the NADH levels were reduced to 10 μM NADH, Ca^{2+} had no effect on the amination reaction. The oxidative deamination reaction of GDH (NAD-GDH) was not affected by addition of Ca^{2+}. For example, with purified GDH, 2.58 and 2.41 μmol NAD were reduced/min·mg protein in the absence and presence of 1 mM Ca^{2+}, respectively.

In animal cells many of the regulatory processes linked to Ca^{2+} are mediated through their interactions with high-affinity Ca^{2+}-binding proteins, such as calmodulin (6). Calmodulin also exists in plant cells (17) but its function is not yet known, except perhaps in chloroplasts (13, 23) and the plasma membrane-enriched microsomal fraction (8). Neither bovine brain calmodulin nor the calmodulin antagonist, trifluoperazine, influenced the activity of purified or mitochondrial derived GDH (data not shown).

Saturation curves for the three substrates of NADH-GDH showed the normal Michaelis-Menten kinetics in the presence of 1 mM Ca^{2+} (Fig. 2). Apparent K_m values of 63 μM for NADH and 2.22 mM for α-KG were calculated from the curves. The apparent K_m for NH_4^+ decreased when the concentration of NADH was lowered: it was 12.5 mM in the presence of 160 μM NADH and 4.76 mM with 10 μM NADH. Thus, the corn shoot-NADH-GDH has kinetic properties similar to the enzyme from *Medicago sativa* (24). With low concentrations of NADH, the apparent V_{max} for NH_4^+ also decreased. In the absence of Ca^{2+}, a marked substrate inhibition was observed with the individual substrates and, hence, the K_m value for each could not be measured.

Estimation of Concentrations of Ca^{2+}, Pyridine Nucleotides, NH_4^+, and Glutamate in Mitochondria Isolated from Corn Shoots. The concentrations of Ca^{2+} in purified mitochondria

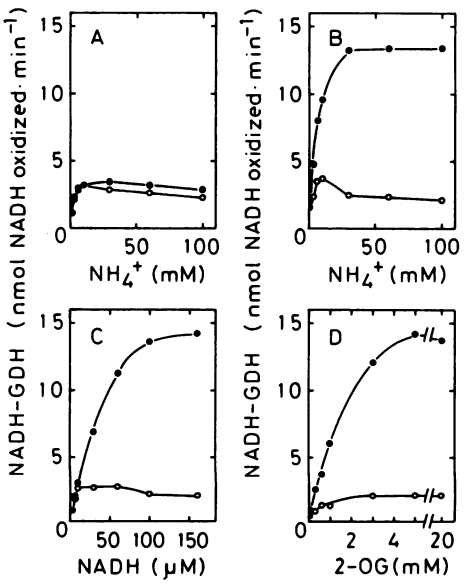

FIG. 2. Substrate saturation curves of the purified NADH-GDH from corn shoots in the presence (●) or absence (○) of 1 mM Ca^{2+}. A, NH_4^+ with 10 μM NADH; B, NH_4^+ with 160 μM NADH; C, NADH; and D, α-KG. In each system the other components were added at the concentrations used in the assay system as described in Figure 1.

Table III. *Determination of Contents of Ca, NAD, NADH Glutamate, Glycine, and Serine in Purified Mitochondria Isolated from the Corn Shoots*

Inhibitors of electron transfer chain, 10 μM rotenone and 5 μM antimycin A, were added to all buffers and Percoll gradient solutions during extraction and purification of mitochondria. The purified mitochondrial pellet was dissolved in a known volume of 0.1 M Bicine-NaOH buffer (pH 8.0) and the suspension was treated with either 0.1 N NaOH or 0.1 N HCl followed by boiling for 2 min for the extraction of NADH or NAD. After neutralization, the pyridine nucleotides were assayed using the cycling method in the presence of alcohol dehydrogenase (19).

Experiment	Ca	NAD	NADH	NH_4	Glu	Gly	Ser
	nmol·mg⁻¹ mitochondrial protein						
I	162	7.22	0.14	5.03	1.21	2.68	1.18
II	149	5.51	0.28	3.64	0.80	1.07	0.65
	mM						
	54	6.37	0.21	4.34	1.01	1.88	0.92

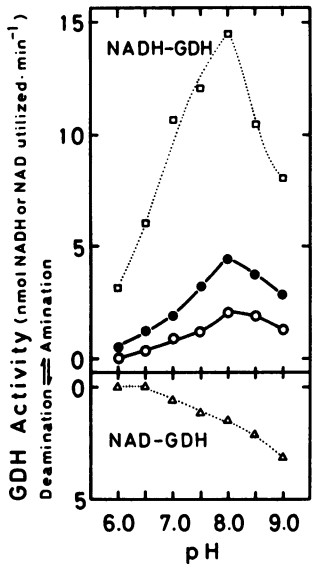

FIG. 3. Simulation of equilibrium of GDH reaction *in vitro* using concentrations of potential substrates estimated to occur in mitochondria of corn shoots as a function of pH. The purified enzyme fraction (1.2 μg protein) was used as the source of enzyme. According to the estimation of substrates (Table III; Ref. 4), the enzyme was incubated with 0.21 mM NADH, 6.4 mM NAD, 5 mM α-KG, 5 mM glutamate, 1 mM $CaCl_2$, and 10 (○) or 50 mM (●) of NH_4Cl in 0.2 M of Tris-Mes buffer at different pH indicated. An oxidation of NADH was observed in all cases. The rate of amination reaction (□) and the deamination reaction (△) were also measured with the standard assay systems as described in "Materials and Methods," except that the Tris-HCl buffers are replaced by the Tris-Mes buffer at different pHs indicated.

from corn shoots was estimated in order to determine its *in vivo* significance (Table III). NADH is also an important factor for the NADH-GDH activity and its activation by Ca^{2+} (Fig. 1), while NAD has been shown to be an inhibitor of the amination reaction (14, 24). Therefore, the concentration of both NAD and NADH were also determined in the purified mitochondria. Purified corn mitochondria contained 149 to 162 nmol Ca/mg protein. When intramitochondrial Ca distibution was determined, after submitochondrial fractionation following the gentle disruption with osmotic shock, about 35% of the total Ca was distributed in the mitochondrial matrix and the remaining 65% with the mitochondrial membrane. If one assumes a matrix water space of 1 μl/mg mitochondrial protein (4, 15), and if the Ca detected in the matrix is all free, the Ca^{2+} concentration in the matrix can be calculated to be 52 to 56 mM. This, together with the fact that the addition of Ca^{2+} had little effect on the NADH-GDH in purified mitochondrial fractions (Table I), suggests that the mitochondrial enzyme is fully activated with respect to Ca^{2+}. The purified mitochondria contained 0.21 nmol NADH and 6.37 nmol NAD/mg mitochondrial protein, respectively. Thus, the concentrations of NADH and NAD in the matrix should be 0.21 and 6.37 mM. The concentration calculated for NADH is higher than the apparent K_m for NADH obtained *in vitro* using the purified GDH in the presence of Ca^{2+} and, hence, an activation by Ca^{2+} would be expected to occur *in vivo*.

Levels of amino acids in the mitochondria were determined by reversed phase chromatography on an Altex Ultrasphere-ODS (5 μM) column as described by Winspear and Oaks (31). Levels of glycine or glutamate in corn mitochondria were 1.88 and 1.01 nmol/mg protein, respectively. NH_4^+ levels were determined after diffusion of NH_4^+ in Conway dishes by the method of McCullough (20). Values for NH_4^+ range from 3.6 to 5.0 mM in corn mitochondria. If NH_4^+ is as diffusible as people claim, then our values are low values (2, 11). Additions of NH_4Cl up to 30 mM had no effect on the respiration of malate. Thus, mitochondrial respiration is several orders of magnitude less sensitive to NH_4^+ than is chloroplast electron transport (11, 21).

To estimate the equilibrium of the GDH reaction in corn mitochondria, purified GDH was incubated in the 0.2 M Tris-Mes buffer at a pH range from 6.0 to 9.0 with the estimated *in vivo* levels of substrate (Fig. 3). According to the results of Bowman *et al.* (4), mitochondria from mung bean contain both α-KG and glutamate in the millimolar range. From these calculations an assay system containing 0.21 mM NADH, 6.4 mM NAD, 5 mM α-KG, 5 mM glutamate, 1 mM Ca^{2+}, and two levels of NH_4^+ (10 and 50 mM) was tested by measuring oxidation of NADH or reduction of NAD. The NH_4^+ levels represent concentrations above or below the K_m for GDH. With these assay conditions, the equilibrium of GDH was in the direction of amination at all pHs tested. As with the standard NADH-GDH assay, a pH optimum of 8.0 was observed. The deamination reaction rate increased with increasing pH over the pH range used. When various concentrations of NH_4^+ (2 to 100 mM) were tested with the reaction system containing all of substrates of GDH at pH 8.0, the equilibrium of the reaction was still in the direction of glutamate production (results not shown).

DISCUSSION

GDH purified from crude mitochondrial preparations of corn shoots is stimulated by Ca^{2+} over a concentration range of 1 μM to 10 mM, when the NADH concentration is in excess of the K_m for NADH. Estimations of the concentrations of Ca and NADH in the mitochondria suggest that NADH-GDH should be in the activated state. Hence NADH-GDH should be in a configuration to support the assimilation of NH_4^+. In a model experiment using pure GDH and all the substrates for NADH- and NAD-GDH at the concentrations estimated to be present in the mitochondria, the equilibrium of the reaction was in the direction of glutamate formation. In recent experiments by Hartmann and Ehmke (11), approximately 14% of the NH_4^+ released from glycine in mitochondria obtained from pea shoots was reassimilated within the mitochondria. This was interpreted by them to be insufficient to consider NADH-GDH as a significant port of entry of NH_4^+. However, no mention was made of optimizing Ca and NADH in their system, hence even this low level of NH_4^+ assimilation should be taken as an indication of the capacity of the mitochondria to assimilate NH_4^+. According to the work by Bergman and Ericson (2), the release of NH_4^+ from glycine occurs at a pH maximum at 8.1 in spinach leaf mitochondria. This pH is almost the same as the optimal pH of NADH-GDH reaction

either in the standard assay system or in the simulated assay system as shown in Figure 3. The conversion of glycine to serine is catalyzed by two enzymes, glycine decarboxylase and serine hydroxymethyltransferase, both of which are localized in the mitochondrial membrane (22). This conversion is also coupled to the formation of NADH (1). Our finding that the loose association of NADH-GDH with the mitochondrial membrane in corn shoots coupled with the generation of both NADH and NH_4^+ in that fraction suggest that GDH could participate in the reassimilation of NH_4^+.

It has been proposed by Miflin and Lea (21) that GS should serve in preference to GDH in the assimilation of NH_4^+ because of its low K_m for NH_4^+. In their scheme, GOGAT allows for the formation of two glutamate molecules from one molecule each of glutamine and α-ketoglutarate. In normal metabolism, one glutamate molecule would be recycled through glutamine and the other would be available for other biosynthetic reactions. In photorespiration, the two glutamate molecules would be required for the synthesis of two glycine molecules, and a third glutamate molecule from somewhere would be required for the regeneration of glutamine. Our results indicate that levels of NH_4^+ in the mitochondria are in the 5 to 10 mM range, and that the mitochondria can tolerate these relatively high concentrations of NH_4^+. At these levels of NH_4^+, there should be a synthesis of glutamate in the mitochondria mediated by GDH. It is our contention that GDH, by virtue of its isolation in mitochondria, can compete successfully with GS for that NH_4^+. A GDH fully saturated with Ca^{2+} and NADH may not be sufficient to assimilate all the NH_4^+ released by the conversion of glycine to serine or, for that matter, produced by the action of nitrate reductase. However, its action would permit the net synthesis of glutamate, glutamine and, subsequently, of other amino acids required to sustain both the GS/GOGAT cycle itself and the enhanced rate of protein synthesis seen in light-treated leaves (5).

LITERATURE CITED

1. Arron GP, MH Spalding, GE Edwards 1979 Stoichiometry of carbon dioxide release and oxygen uptake during glycine oxidation in mitochondria isolated from spinach (*Spinacia oleracea*) leaves. Biochem J 184: 457–460
2. Bergman A, I Ericson 1983 Effects of pH, NADH, succinate and malate on the oxidation of glycine in spinach leaf mitochondria. Physiol Plant 59: 421–427
3. Bird IF, MJ Cornelius, AJ Keys, CP Whittingham 1972 Oxidation and phosphorylation associated with the conversion of glycine to serine. Phytochemistry 11: 1587–1594
4. Bowman EJ, H Ikuma, HJ Stein 1976 Critic acid cycle activity in mitochondria isolated from mung bean hypcotyls. Plant Physiol 58: 426–432
5. Buchanan BB 1980 Role of light in the regulation of chloroplast enzymes. Annu Rev Plant Physiol 31: 239–298
6. Cheung WY 1980 Calmodulin plays a pivotal role in cellular regulation. Science 207: 19–27
7. Chou K-H, We Splittstoesser 1972 Glutamate dehydrogenase from pumpkin cotyledons. Characterization and isoenzymes. Plant Physiol 49: 550–554
8. Dieter P, D Marme 1983 The effect of calmodulin and far-red light on the kinetic properties of the mitochondrial and microsomal calcium-ion transport system from corn. Planta 159: 277–281
9. Douce R, EL Christensen, WD Bonner J 1972 Preparation of intact mitochondria. Biochim Biophys Acta 275: 148–160
10. Furuhashi K, Y Takahashi 1982 Control of glutamate dehydrogenase from green tobacco callus mitochondria by Ca^{2+} and pH. Plant Cell Physiol 23: 179–184
11. Hartmann T, A Ehmke 1980 Role of mitochondrial glutamate dehydrogenase in the reassimilation of ammonia produced by glycine-serine transformation. Planta 149: 207–208
12. Hatch MD 1978 A simple spectrophotometric assay for fumarate hydratase in crude tissue extracts. Anal Biochem 85: 271–275
13. Jarrett HW, CJ Brown, CC Black, MJ Cormier 1982 Evidence that calmodulin is in the chloroplast of peas and serves a regulatory role in photosynthesis. J Biol Chem 257: 13795–13804
14. Joy KW 1973 Control of glutamate dehydrogenase from *Pisum sativum* roots. Phytochemistry 12: 1031–1040
15. Lanoue K, WJ Nicklas, JR Williamson 1970 Control of citric acid cycle activity in rat heart mitochondria. J Biol Chem 245: 102–111
16. Lowry OH, NJ Rosebrough, AL Farr, RJ Randall 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
17. Matsumoto H, M Tanigawa, T Yamaya 1983 Calmodulin-like activity associated with chromatin from pea buds. Plant Cell Physiol 24: 593–602
18. Matsumoto H, T. Yamaya 1981 Increase in alkaline phosphatase activity in cucumber roots during calcium starvation. Plant Cell Physiol 22: 1137–1140
19. Matsumura H, S Miyachi 1980 Cycling assay for nicotinamide adenine dinucleotides. Methods Enzymol 69: 465–470
20. McCullough H 1967 The determination of ammonia in whole blood by a direct colorimetric assay. Clin Chim Acta 17: 297–304
21. Miflin BJ, PJ Lea 1977 Amino acid metabolism. Annu Rev Plant Physiol 28: 299–329
22. Moore AL, C Jackson, B Halliwell, JE Dench, DO Hall 1977 Intamitochondrial localization of glycine decarboxylase in spinach leaves. Biochem Biophys Res Commun 78: 483–491
23. Muto S 1982 Distribution of calmodulin within wheat leaf cells. FEBS Lett 147: 161–164
24. Nagel M, T Hartmann 1980 Glutamate dehydrogenase from *Medicago sativa* L., Purification and comparative kinetic studies of the organ-specific multiple forms. Z Naturforsch 35c: 406–415
25. Nauen W, T Hartmann 1980 Glutamate dehydrogenase from *Pisum sativum* L., Localization of the multiple forms and of glutamate formation in isolated mitochondria. Planta 148: 7–16
26. Nishimura M, R Douce, T Akazawa 1982 Isolation and characterization of metabolically competent mitochondria from spinach leaf protoplasts. Plant Physiol 69: 916–920
27. Oaks A, I Stulen, K Jones, MJ Winspear, S Misra, IL Boesel 1980 Enzymes of nitrogen assimilation in maize roots. Planta 148: 477–484
28. Priestley DA, J Bruinsma 1982 Sensitivity to drying in vitro of enzymes in mitochondrial subfractions from *Vicia faba* seed. Physiol Plant 56: 303–311
29. Quail PH 1979 Plant cell fractionation. Annu Rev Plant Physiol 30: 425–484
30. Suzuki A, P Gadal, A Oaks 1981 Intracellular distribution of enzymes associated with nitrogen assimilation in roots. Planta 151: 457–461
31. Winspear MJ, A Oaks 1983 Automated pre-column amino acid analysis by reversed phase high performance liquid chromatography. J Chromatogr 270: 378–382
32. Yamaya T, A Oaks, H Matsumoto 1984 Stimulation of mitochondrial calcium uptake by light during growth of corn shoots. Plant Physiol 75: 773–777