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Physical and Kinetic Properties of the Nicotinamide Adenine Dinucleotide-specific Glutamate Dehydrogenase Purified from *Chlorella sorokiniana* [1]

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ABSTRACT

The nicotinamide adenine dinucleotide-specific glutamate dehydrogenase (L-glutamate:NAD^+ oxidoreductase, EC 1.4.1.2) of *Chlorella sorokiniana* was purified 1,000-fold to electrophoretic homogeneity. The native enzyme was shown to have a molecular weight of 180,000 and to be composed of four identical subunits with a molecular weight of 45,000. The N-terminal amino acid was determined to be lysine. The pH optima for the aminating and deaminating reactions were approximately 8 and 9, respectively. The K_m values for α-ketoglutarate, NADH, NH_4^+, NAD^+, and L-glutamate were 2 mM, 0.15 mM, 40 mM, 0.15 mM, and 60 mM, respectively. Whereas the K_m for α-ketoglutarate and L-glutamate increased 10-fold, 1 pH unit above or below the pH optima for the aminating or deaminating reactions, respectively, the K_m values for NADH and NAD^+ were independent of change in pH from 7 to 9.6. By initial velocity, product inhibition, and equilibrium substrate exchange studies, the kinetic mechanism of enzyme was shown to be consistent with a bi uni uni uni ping-pong addition sequence. Although this kinetic mechanism differs from that reported for any other glutamate dehydrogenase, the chemical mechanism still appears to involve the formation of a Schiff base between α-ketoglutarate and an ε-amino group of a lysine residue in the enzyme. The physical, chemical, and kinetic properties of this enzyme differ greatly from those reported for the NH_4^+-inducible glutamate dehydrogenase in this organism.

Current evidence (28, 33) indicates that the coupled reactions of glutamine synthetase and glutamate synthase might be the primary route of ammonium assimilation, via glutamate, into higher plants and green algae. If so, what is the function of the various multiple forms (*i.e.* isozymes) of glutamate dehydrogenase which have different coenzyme specificities and/or different intracellular locations in higher plants (3, 11, 14, 25, 27, 29) and green algae (15, 24, 38), particularly those (3, 25, 27, 34, 41) whose levels increase dramatically *in vivo* in response to ammonia?

In this laboratory, the metabolic roles and the molecular mechanism(s) regulating the levels of an ammonium-inducible NADP-GDH[5] and a constitutive NAD-GDH are being studied in synchronous cultures of *Chlorella sorokiniana*. Talley *et al.* (41) and Israel *et al.* (23, 24) have reported that the activities of both glutamate dehydrogenase isozymes change dramatically during the cell cycle and during shifts in the nitrogen nutritional status of this organism.

The purpose of the present study was to purify the NAD-GDH from *C. sorokiniana* to determine whether this isozyme is a distinct molecular species from the NADP-GDH (19) of this same organism, and to begin an investigation of its metabolic role by examining certain aspects of its kinetic and chemical mechanism.

MATERIALS AND METHODS

Culture Conditions and Harvest Procedure. *C. sorokiniana* was cultured in nitrate-containing medium with 4% CO_2-air at 38.5 C in a 35-liter chamber as previously described (37). When the culture turbidity (*i.e.* *A* at 550 nm, 1.3-cm-diameter colorimeter tube, Bausch & Lomb model 340 spectrophotometer) reached 2 to 2.1, approximately 110 g fresh wt of cells in 30 liters of suspension were harvested in a Sharples centrifuge (26,000*g*) at 4 C. The cells were washed three times in 0.01 M Tris-HCl (pH 7.9), resuspended in 2 volumes of 0.1 M Tris-HCl, and frozen at −20 C.

Enzyme and Total Protein Assays. The NAD-GDH activity was assayed with a slight modification of the procedure of Israel *et al.* (23). The assay mixture was modified to contain 50 mM Tris-HCl (pH 7.9) at 38.5 C. Total protein concentration was measured by the method of Lowry *et al.* (31).

Purification of NAD-GDH. Approximately 220 g fresh wt of frozen cells were thawed, ruptured by passage through a French pressure cell, and the resulting homogenate was centrifuged for 1 hr at 100,000*g*. The 100,000*g* supernatant was heated at 65 C for 25 min, cooled in an ice bath, and then centrifuged at 27,000*g*. The heat-treated supernatant was brought to 20% $(NH_4)_2SO_4$ saturation, centrifuged, and the supernatant was adjusted to 55% saturation with this salt. The protein precipitate was recovered by centrifugation and dissolved in 0.1 M Tris-HCl buffer (pH 7.9). This solution was applied to a column (5 × 100 cm) of Sephadex G-200 equilibrated and eluted with the buffer. The fractions containing NAD-GDH activity were combined, and applied directly to a column (2.5 × 40 cm) of Whatman DE52 anion exchange cellulose equilibrated with the same buffer. The column was washed with the buffer, and the enzyme was then eluted with a 1,000-ml linear gradient from 0.1 to 0.4 M NaCl. The fractions containing NAD-GDH activity were concentrated by ultrafiltration, and then dialyzed for 12 hr against the Tris-HCl buffer. Preparative gel electrophoresis was performed (28 mamp) in a 1.2-

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[5] Abbreviations: NADP-GDH: nicotinamide adenine dinucleotide phosphate-specific glutamate dehydrogenase; NAD-GDH: nicotinamide adenine dinucleotide-specific glutamate dehydrogenase; α-CBZ-: α-carbobenzyloxy-.

cm 7% acrylamide separating gel and a 2.5-cm 2.5% acrylamide stacking gel in the P2-320 column in a Canalco Prep-Disc apparatus.

At the different purification steps, the homogeneity of the NAD-GDH was examined by analytical disc electrophoresis (12) followed by staining with Coomassie blue.

Determination of Molecular Wt of NAD-GDH and Its Subunit. By use of a column (1.5 × 100 cm) of Sephadex G-200 equilibrated with 0.1 M Tris-HCl (pH 7.9) and calibrated with protein standards, the mol wt of the purified NAD-GDH was estimated by the method of Andrews (2). The mol wt of the native enzyme was also estimated with a column (1.5 × 100 cm) of Sepharose-6B which was prepared and calibrated as described above.

The mol wt of the subunit of the dissociated NAD-GDH was estimated by SDS-polyacrylamide gel electrophoresis as described by Weber and Osborn (42).

Amino Acid Analysis. The amino acid composition of the purified NAD-GDH was partially determined with a Beckman model 121 automatic amino acid analyzer. The enzyme was hydrolyzed in 6 N HCl under N_2 atmosphere for 24 hr at 110 C, and then dried *in vacuo*. The concentration of serine and threonine was determined by hydrolyzing enzyme samples for 24, 48, and 72 hr and extrapolating amino acid content back to zero hydrolysis time. Prior to acid hydrolysis, the enzyme which was to be analyzed for cysteine and methionine content was oxidized with performic acid by the method of Devenyi and Gergely (13). Tryptophan and tyrosine content of the enzyme was measured by the method of Bencze and Schmid (7). The number of methionine residues was confirmed by cyanogen bromide cleavage, and chromatographic separation of the resultant peptides (13).

The dansylation method of Gray (18) was used to determine the N-terminal amino acid of the purified NAD-GDH. The standard dansylated amino acids were separated on polyamide layer sheets with the solvent systems described by Woods and Wang (43).

NAD-GDH Substrate Exchange at Equilibrium. The method of Silverstein and Boyer (39) was used to test equilibrium substrate exchange. The reaction mixture contained 0.075 mM NADH, 2 mM thio-NAD^+, 25 mM α-ketoglutarate, and 50 mM Tris-HCl (pH 7.9, 38.5 C) in different combinations described later. The oxidation of NADH and reduction of thio-NAD^+ were measured at 340 nm and 400 nm, respectively.

Procedure for Product Inhibition Studies. Product inhibition of enzyme activity was tested at NAD^+ and glutamate concentrations of 0.2, 0.5, or 1 mM, and 5, 10, or 20 mM, respectively. The concentrations of NH_4^+ and α-ketoglutarate tested for inhibition were 50, 100, or 200 mM, and 0.2, 0.5, or 1 mM, respectively. The concentration described as saturating was 3 to 10 times the K_m of the substrate while the concentration designated to be unsaturating was equal to the K_m of the substrate. The reactions were initiated by addition of enzyme.

Chemical Inactivation of NAD-GDH. The incubation mixture used to test the effect of substrates on inactivation of NAD-GDH by methylacetimidate was prepared as follows: 80 μg of pure NAD-GDH in 100 μl of 0.1 M Tris-HCl (pH 8.5); 100 μl of substrates at four times their desired final concentration in 0.1 M Tris-HCl (pH 8.5); 200 μl of 10^{-7} M methylacetimidate in 0.1 M Tris-HCl (pH 8.5). In the 400-μl final incubation mixture, the mole ratio of enzyme to methylacetimidate was approximately 1:500. The solution containing the substrate (s) to be tested was added to the enzyme before the methylacetimidate.

The procedure described for inactivation by methylacetimidate was also used for cyanide inactivation studies. The concentration of KCN in the final mixture was 50 mM.

A similar procedure was repeated with the substitution of pyridoxal-5'-P as the inactivating agent. The reaction was performed in the dark. Reactivation of the pyridoxal-5'-P-treated enzyme was performed by dialysis of 1 ml against 3 liters of 0.1 M Tris-HCl (pH 7.9) at 22 C in the dark for 12 hr (1).

Photomediated inactivation by pyridoxal-5'-P was tested by placement of a 150-w light bulb at a distance of 5 cm from an enzyme-pyridoxal-5'-P solution for 30 min. An identical solution containing only the NAD-GDH was used as a control. After dialysis, both samples were assayed for enzyme activity.

Identification of a Dansylated Reduced Schiff Base Intermediate of the NAD-GDH. Inactivation of the purified NAD-GDH was performed by incubation of 0.2 mg of enzyme with 500 μmol of α-[5-^{14}C]ketoglutarate (1 μCi/mol) in the presence of 10 nmol of $NaBH_4$ by the method of Rasched *et al.* (36). The $NaBH_4$ and radioactive α-ketoglutarate were dissolved in 2 ml of 0.1 M Tris-HCl (pH 8) and added to the enzyme solution sequentially in 0.1-ml aliquots starting with α-ketoglutarate. After 2 hr, the enzyme was inactivated by approximately 65%. The inactivated enzyme was dialyzed against 10^5 volumes of deionized H_2O, and then lyophilized. The enzyme was dissolved in 0.2 ml of deionized H_2O, and 0.1 ml of this solution was subjected to dansylation and then acid-hydrolyzed as described earlier for amino acid analysis. The remaining 0.1 ml was taken to dryness by flash evaporation, and the enzyme acid-hydrolyzed. The amino acids in this latter hydrolysate were then dansylated. Since N-α-dansyl-N-ε-glutaryl-L-lysine was not available commercially as a standard, it was synthesized by reduction of the ε-amino adduct of N-α-CBZ-L-lysine and α-ketoglutarate.

Chemicals. The thio-NAD^+ was a gift from B. M. Anderson, V.P.I. and S.U. α-[5-^{14}C]Ketoglutarate was purchased from New England Nuclear; dansyl amino acids, dansyl chloride, N-α-CBZ-L-lysine, and BSA from Sigma Chemical Co.; aldolase, chymotrypsinogen, ribonuclease, and ovalbumin from Pharmacia Fine Chemicals. Other reagents were obtained from various sources and were of highest purity available.

TABLE I
PURIFICATION OF NAD-GLUTAMATE DEHYDROGENASE FROM CHLORELLA SOROKINIANA

	Purification Step	Total Units[1]	Total Protein (mg)	S.A.[2]	Recovery (%)	Fold Purification
	100,000 g Supernatant	2,500	12,700	0.20	100	1
1.	Heat (65 C, 25 min) Treatment	2,500	4,400	0.60	100	3
2.	Ammonium Sulfate Ppt. (20-55%)	1,800	978	1.80	72	9
3.	Sephadex G-200 Gel Filtration	1,440	130	11	58	55
4.	DE-52 Ion Exchange Chromatography	1,200	24	50	48	250
5.	Preparative Gel Electrophoresis	600	3	200	24	1000

[1] One unit is defined as the enzyme necessary to oxidize 1 μmol of NADH/min at 38.5 C

[2] Specific activity is defined as units/mg protein

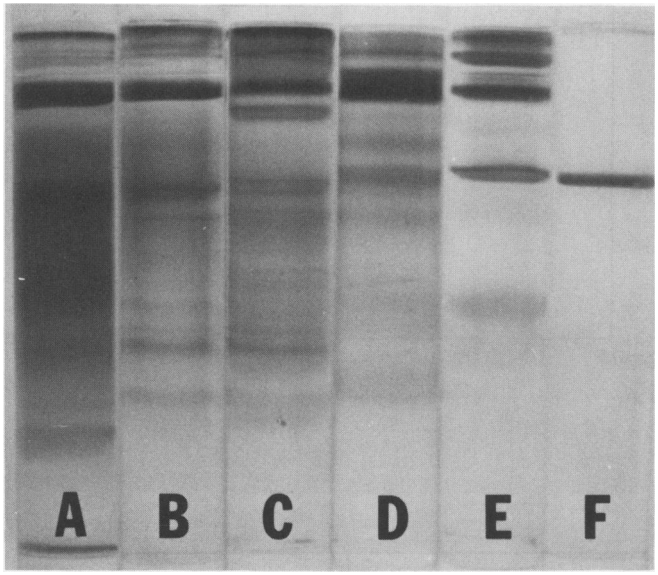

FIG. 1. Polyacrylamide disc gels of *C. sorokiniana* NAD-glutamate dehydrogenase at each stage of purification. A: 100,000*g* supernatant; B: heat treatment (65 C for 25 min); C: 20 to 55% ammonium sulfate; D: Sephadex G-200 gel filtration; E: DE52 ion exchange chromatography; F: preparative disc gel electrophoresis. The gels were 7% acrylamide and received 50 to 75 μg of total protein. After electrophoresis, the gels were stained with Coomassie blue.

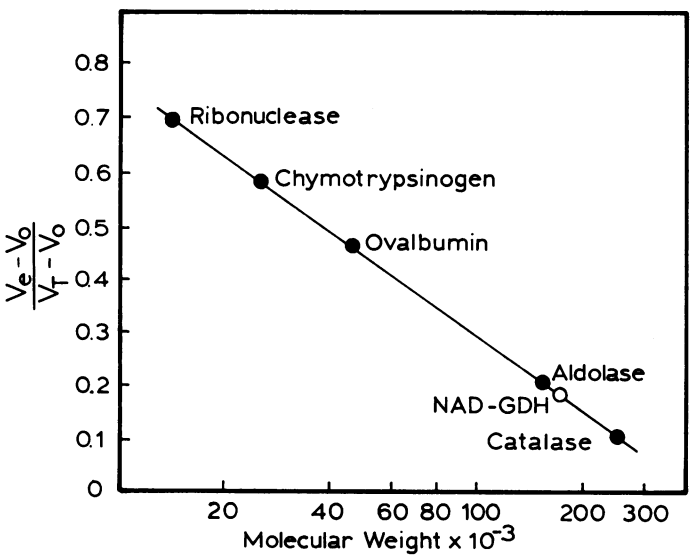

FIG. 2. Determination of mol wt of NAD-glutamate dehydrogenase from *C. sorokiniana* by gel filtration with a column (1.5 × 100 cm) of Sephadex G-200 eluted with 0.1 M Tris-HCl (pH 7.9) at 22 C. V_0: void volume; V_e: elution volume; V_T: column bed volume.

RESULTS

Purification of the NAD-GDH. Beginning with a 100,000*g* supernatant from *Chlorella* cells cultured in nitrate-containing medium, the NAD-GDH was purified to electrophoretic homogeneity in five steps (Table I). Analytical disc gel electrophoresis was used to evaluate the degree of homogeneity of the enzyme at different stages of purification (Fig. 1). The heat-stable nature of the enzyme facilitated rapid purification; however, a large loss of activity occurred during preparative gel electrophoresis. Addition of DTT (1–10 mM) did not prevent or reverse the loss of activity.

Molecular wt of NAD-GDH and Its Subunit. From the elution volumes of protein standards and of the purified NAD-GDH from a Sephadex G-200 column, the mol wt of the native enzyme was estimated to be 180,000 (Fig. 2). With a similar procedure and a column of Sepharose 6B, an identical mol wt was obtained for this enzyme. By comparison of the electrophoretic mobility of protein standards with that of the purified NAD-GDH during

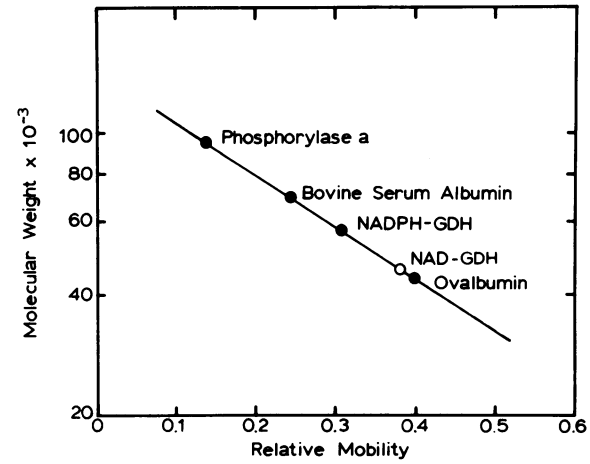

FIG. 3. Determination of the mol wt of the subunit of NAD-glutamate dehydrogenase from *C. sorokiniana* by SDS-polyacrylamide gel electrophoresis.

TABLE II
AMINO ACID COMPOSITION OF NAD-GLUTAMATE DEHYDROGENASE FROM CHLORELLA SOROKINIANA

Amino Acid	Residues/Subunit[1] (Nearest Integer)
Lys	27
His	8
Arg	6
Cys	6
Asp + Asn	48
Met	6
Thr	30
Ser	74
Glu + Gln	80
Pro	38
Gly	35
Ala	16
Val	8
Leu	14
Ilu	7
Phe	6
Tyr	6
Trp	6

[1]Molecular weight of the NAD-GDH subunit, approx. 45,000 daltons

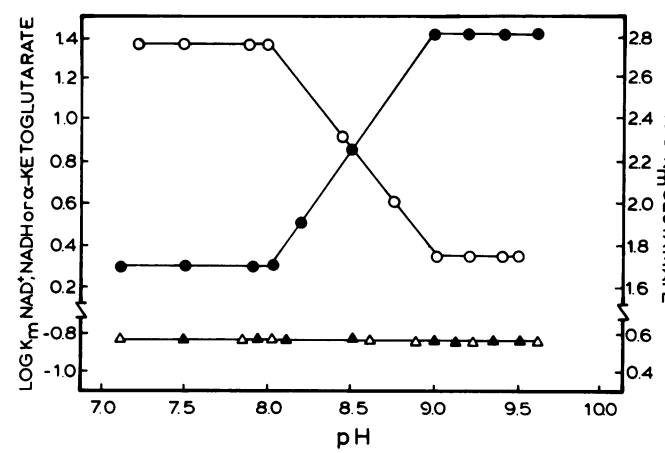

FIG. 4. Effect of pH on the K_m for α-ketoglutarate (●), NADH (▲), NAD^+ (△), and glutamate (○) of the NAD-glutamate dehydrogenase from *C. sorokiniana.* The K_m values were determined by linear regression analysis of experimental data. The log K_m was calculated from the Michaelis constants (mM).

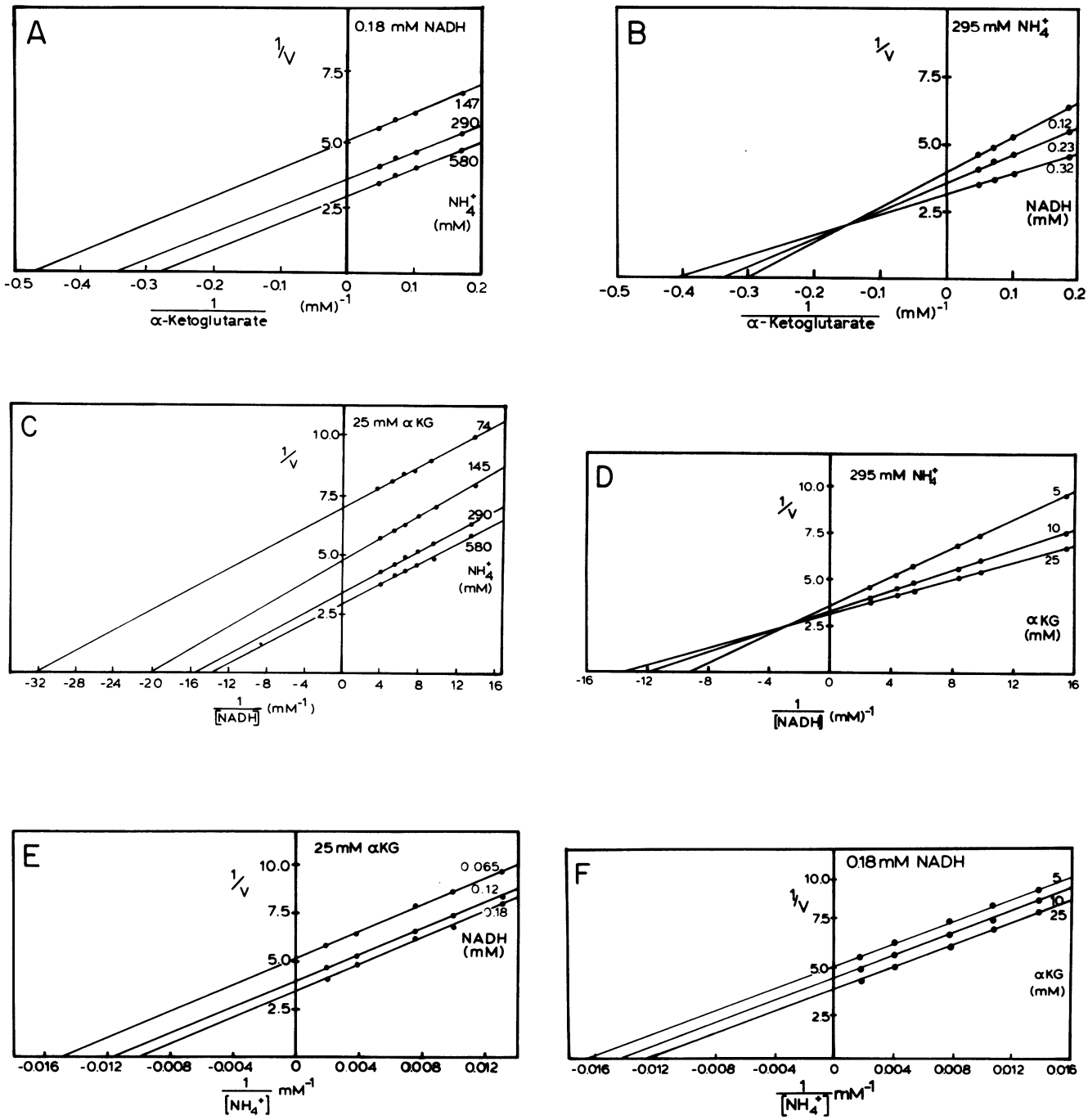

FIG. 5. A, B, C, D, E, F: double reciprocal plots of initial velocities at different concentrations of one substrate, constant concentration of a second substrate, and fixed levels of a third substrate for the reaction of the NAD-specific glutamate dehydrogenase from *C. sorokiniana*. Concentration of substrates: A, α-ketoglutarate varied, NADH constant, NH_4 fixed; B: α-ketoglutarate varied, NH_4^+ constant, NADH fixed; C: NADH varied; α-ketoglutarate constant; NH_4^+ fixed; D: NADH varied; NH_4^+ constant; α-ketoglutarate fixed; E: NH_4^+ varied; α-ketoglutarate constant; NADH fixed; F: NH_4^+ varied; NADH constant; α-ketoglutarate fixed. Each assay contained 1 μg of purified enzyme and each point on the graphs is the average of four spectrophotometric assays. Slope and intercept values were calculated by linear regression analysis.

SDS-polyacrylamide gel electrophoresis, the dissociated enzyme migrated as a single mol wt species of 45,000 daltons (Fig. 3).

Amino Acid Composition of the NAD-GDH. The amino acid composition of the NAD-GDH is shown in Table II.

Seven peptides were produced by cyanogen bromide cleavage of the purified NAD-GDH. This number of peptides would be the expected cleavage products of an enzyme containing six methionine residues. If the subunits of this enzyme were not identical, even if different subunits contained six methionines, it is very unlikely that the peptides produced by cyanogen bromide cleavage would be indistinguishable by both the electrophoretic and chromatographic procedures used in the present study.

The only dansyl derivative released, after acid hydrolysis of the dansylated NAD-GDH, was identified as N-α-N-ε-didansyl lysine by co-chromatography with a standard through two dimensions of the solvent system of Woods and Wang (43). Thus, lysine is the N-terminal amino acid of the NAD-GDH.

pH Optima and Michaelis Constants for NAD-GDH. The pH optima for the aminating and deaminating reactions of the purified NAD-GDH were observed to be approximately 8 and 9.1, respectively.

The K_m values for the substrates were measured at the pH

optimum for that reaction. The K_m values for α-ketoglutarate, NADH, NH_4^+, glutamate, and NAD^+ were determined to be 2 mM, 0.15 mM, 40 mM, 60 mM, and 0.15 mM, respectively.

When the effect of pH on the K_m was investigated, it was found that the K_m values for NADH and NAD^+ were unaltered between pH 7.2 and 9.7. However, the K_m for glutamate decreased from 630 mM to 60 mM and the K_m for α-ketoglutarate increased from 2 mM to 25 mM over the pH range from 8 to 9 (Fig. 4). A pH-dependent effect was seen on the V_{max} of only the aminating reaction.

Kinetic Mechanism of NAD-GDH. The family of double reciprocal plots (Fig. 5 A, B, C, D, E, and F) obtained from initial velocity studies with the purified NAD-GDH describe a bi uni uni uni ping-pong kinetic mechanism (Fig. 6). All substrate saturation curves followed Michaelis-Menten kinetics in these and other experiments performed at higher substrate concentrations. Although these studies unambiguously define NH_4^+ to be the third substrate added, the addition sequence of α-ketoglutarate and NADH could not be ascertained by initial velocity studies. However, the data obtained from product inhibition studies of the aminating (Table III) and deaminating (Table IV) reactions were also consistent with the pattern of inhibition predicted for the bi uni uni uni ping-pong kinetic mechanism (Fig. 6).

Thio-NAD^+ was shown to be a reducible substrate of the purified NAD-GDH with a K_m (0.2 mM) only slightly higher than that of NAD^+. Because this reduced analog absorbs at 400 nm rather than at 340 nm, it was used to help establish the positions of NADH and NAD^+ in the proposed kinetic mechanism. When equilibrium was reached, in the absence of NH_4^+, equimolar amounts of NADH and thio-NAD^+ had been oxidized and reduced, respectively (Table V). This hydride-ion exchange required

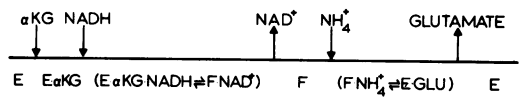

FIG. 6. Sequence of substrate addition for the reaction of the NAD-glutamate dehydrogenase of *C. sorokiniana*. E: free enzyme; αKG: α-ketoglutarate; F: NAD-glutamate dehydrogenase-α-ketoglutarate intermediate; Glu: glutamate.

TABLE III
PRODUCT INHIBITION PATTERN OF THE AMINATING REACTION OF NAD-SPECIFIC GLUTAMATE DEHYDROGENASE FROM CHLORELLA SOROKINIANA

Product Inhibitor	Varied Substrates	Saturating Substrates	Unsaturating Substrates	Type of Inhibition
NAD^+	α-KG[1]	NADH	NH_4^+	Uncompetitive
NAD^+	α-KG	NADH, NH_4^+	---	No Inhibition[2]
NAD^+	α-KG	---	NADH, NH_4^+	Mixed Type
NAD^+	NADH	α-KG, NH_4^+	---	No Inhibition[2]
NAD^+	NADH	NH_4^+	α-KG	Mixed Type
NAD^+	NADH	---	α-KG, NH_4^+	Mixed Type
NAD^+	NH_4^+	α-KG, NADH	---	Competitive
NAD^+	NH_4^+	NADH	α-KG	Competitive
NAD^+	NH_4^+	---	α-KG, NADH	Competitive
Glutamate	α-KG	---	NADH, NH_4^+	Competitive
Glutamate	α-KG	NADH, NH_4^+	---	Competitive
Glutamate	α-KG	NH_4^+	NADH	Competitive
Glutamate	NADH	α-KG	NH_4^+	No Inhibition[2]
Glutamate	NADH	NH_4^+	α-KG	Mixed Type
Glutamate	NADH	---	α-KG, NH_4^+	Mixed Type
Glutamate	NH_4^+	---	α-KG, NADH	Mixed Type
Glutamate	NH_4^+	α-KG	NADH	No Inhibition[2]
Glutamate	NH_4^+	NADH	α-KG	Mixed Type

[1] α-Ketoglutarate

[2] No slope or intercept variation was measurable

TABLE IV
PRODUCT INHIBITION PATTERN OF THE DEAMINATING REACTION OF NAD-GLUTAMATE DEHYDROGENASE FROM CHLORELLA SOROKINIANA

Product Inhibitor	Varied Substrate	Saturating Substrate	Unsaturating Substrate	Type of Inhibition
α-KG[1]	Glutamate	---	NAD^+	Competitive
α-KG	Glutamate	NAD^+	---	Competitive
α-KG	NAD^+	---	Glutamate	Uncompetitive
α-KG	NAD^+	Glutamate	---	No Inhibition[2]
NH_4^+	Glutamate	---	NAD^+	Mixed Type
NH_4^+	Glutamate	NAD^+	---	No Inhibition[2]
NH_4^+	NAD^+	---	Glutamate	Competitive
NH_4^+	NAD^+	Glutamate	---	Competitive

[1] α-Ketoglutarate

[2] No slope or intercept effect was measurable

both α-ketoglutarate and NAD-GDH, and the rate at which equilibrium was reached was proportional to enzyme concentration.

Search for Possible Modifiers of NAD-GDH Activity. The following compounds were tested for their ability to activate or to inhibit the activity of the purified NAD-GDH: L-alanine, L-glutamine, L-leucine, L-histidine, L-proline, carbamyl phosphate, guanine, guanosine, GMP, GDP, GTP, adenine, adenosine, AMP, ADP, ATP, cytosine, CMP, CDP, CTP, thymine, TMP, TDP, TTP, and UMP. All potential modifiers were tested at 1 and 19 mM concentrations at the pH optima of the amination and deamination reactions. Only ADP had any effect. Both the aminating and deaminating reactions were inhibited competitively by ADP *versus* the pyridine nucleotide coenzyme with a K_i of 10 mM.

Chemical Modification of NAD-GDH. Selective conversion of amino groups to imidines has been described for a number of proteins by use of alkyl imidoesters (32). Methylacetimidate reacts with the α-amino group of N-terminal amino acids, but more rapidly with the ε-amino group of lysine residues. As would be expected, if lysine were involved in catalytic activity of the NAD-GDH, the enzyme was rapidly inactivated by this modifying agent (Fig. 7).

Incubation of the NAD-GDH with KCN in the absence of substrates had no effect on activity (Fig. 8). When the enzyme was incubated with KCN and 20 mM α-ketoglutarate, or KCN and 60 mM L-glutamate, enzyme activity was rapidly lost. The α-ketoglutarate-treated enzyme exhibited the greater loss in activity. These data are consistent with the formation of a Schiff base intermediate (10) in the enzymic reaction. When NADH was included in a mixture, containing both KCN and α-ketoglutarate, NAD-GDH was inactivated to a lesser degree than with α-ketoglutarate alone. The chemical events responsible for this latter observation are unclear.

When incubated with pyridoxal-5'-P in the absence of substrates, NAD-GDH activity was rapidly lost (Fig. 9). The protection of enzyme activity was observed to be proportional to the concentration of α-ketoglutarate, suggesting that pyridoxal-5'-P and α-ketoglutarate compete for the same binding site (1). By itself NADH offered no protection against enzyme inactivation; however, in conjunction with α-ketoglutarate, NAD-GDH activity was completely protected. The inactivation by pyridoxal-5'-P was reversible by dialysis. However, when a sample of the treated enzyme was exposed to light, as described by Hucho *et al.* (22), the inactivation could not be reversed by dialysis.

The inactivation of bovine GDH by $NaBH_4$ and α-ketoglutarate is assumed to be the result of reduction of a Schiff base intermediate (1, 36). When NAD-GDH was inactivated by $NaBH_4$ in the presence of α-[5-^{14}C]ketoglutarate, and then dansylated prior to acid hydrolysis, the only dansyl derivative detected after acid hydrolysis was N-α-N-ε-didansyl lysine. This lysine derivative, formed by dansylation of the N-terminal amino acid, was not

TABLE V
SUBSTRATE EXCHANGE OF PURIFIED NAD-GLUTAMATE DEHYDROGENASE AT EQUILIBRIUM: NADH AND THIO-NAD$^+$

Sample	Wavelength (nm)	ΔAbsorbance	μmol Nucleotide Reduced or Oxidized	Rate of Exchange μmol/min	Specific Exchange μmol/min/mg enzyme
200 μg GDH + T-NAD*	400	0	---	---	---
200 μg GDH + T-NAD + NADH	400	0	---	---	---
NADH + T-NAD + α-KG	400	0	---	---	---
200 μg GDH + NADH + T-NAD + α-KG	400	0.92, 0.915	0.077	0.035	0.175
100 μg GDH + NADH + T-NAD + α-KG	400	0.90, 0.90	0.075	0.016	0.166
200 μg GDH + NADH	340	0	---	---	---
200 μg GDH + NADH + T-NAD	340	0	---	---	---
200 μg GDH + NADH + α-KG	340	0	---	---	---
200 μg GDH + NADH + T-NAD + α-KG	340	-0.465,-0.46	0.074	0.032	0.161
100 μg GDH + NADH + T-NAD + α-KG	340	-0.45, -0.46	0.072	0.016	0.160

*Thionicotinamide adenine dinucleotide

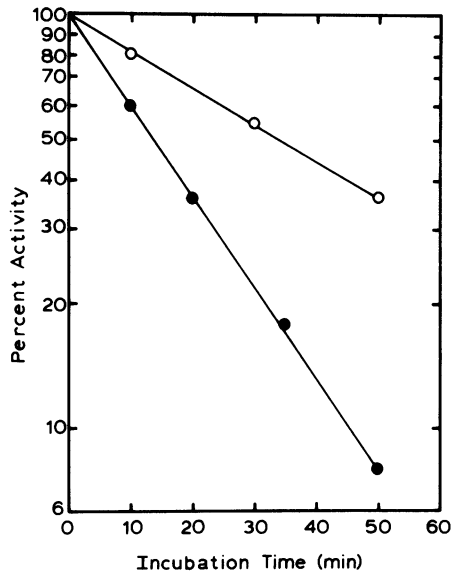

FIG. 7. Inactivation of the NAD-glutamate dehydrogenase from *C. sorokiniana* by 0.1 M methylacetimidate in the presence of 20 mM α-ketoglutarate (○), 20 mM α-ketoglutarate plus 0.3 mM NADH (○), 60 mM glutamate (●), 0.3 mM NADH (●), 0.3 mM NAD$^+$ (●), or no additions (●). Enzyme activity was identical in all samples at zero incubation time. Per cent activity is per cent of initial enzyme activity.

radioactive. When the enzyme was first hydrolyzed, and the hydrolysis products subjected to dansylation, only one radioactive compound could be detected. This compound co-chromatographed with the standard sample of N-α-dansyl-N-ε-glutaryl-L-lysine. The tentative conclusion is that this radioactive compound is a reduced Schiff base intermediate formed between α-ketoglutarate and the ε-amino group of lysine at the catalytic site of the enzyme.

DISCUSSION

From physical studies with the NAD-GDH in *C. sorokiniana*, we concluded that the native enzyme has a mol wt of 180,000 and is composed of four identical subunits with a mol wt of 45,000. Although all glutamate dehydrogenases studied so far are composed of identical subunits (40), the NAD-GDH from *Neurospora* (40) and the NAD-GDH from *C. sorokiniana* are the only glutamate dehydrogenases reported to be composed of four instead of six identical subunits. The NAD-GDH from *C. pyrenoidosa* was

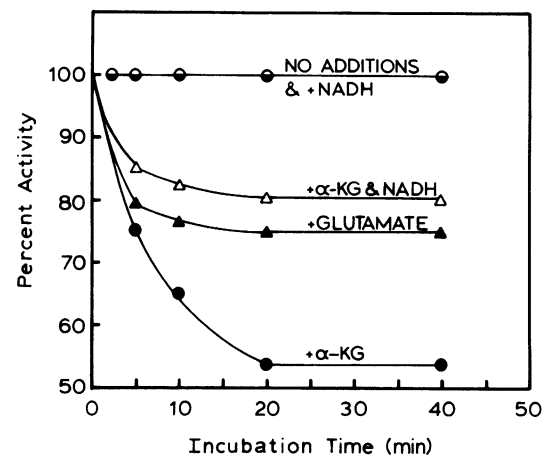

FIG. 8. Inactivation of the NAD-glutamate dehydrogenase from *C. sorokiniana* by 50 mM KCN in the presence of 20 mM α-ketoglutarate (●), 60 mM glutamate (▲), 20 mM α-ketoglutarate plus 0.3 mM NADH (△), 0.3 mM NADH (◒), or no additions (◒).

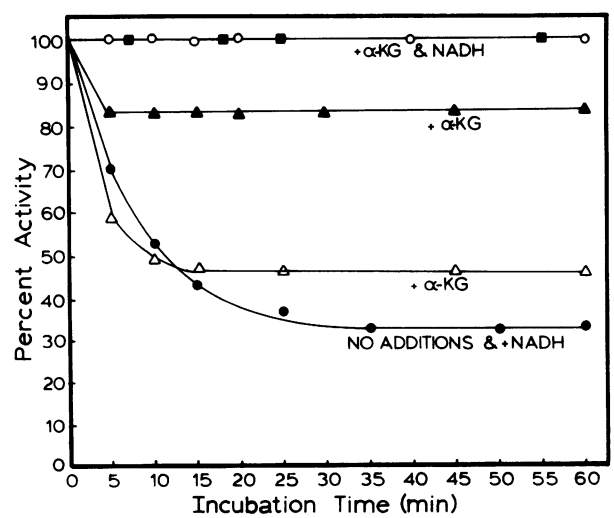

FIG. 9. Inactivation of NAD-glutamate dehydrogenase from *C. sorokiniana* by 1 mM pyridoxal-5′-phosphate in the presence of 5 mM α-ketoglutarate (△), 20 mM α-ketoglutarate (▲), 0.3 mM NADH (●), 20 mM α-ketoglutarate plus 0.3 mM NADH (○), or no additions (●). Enzyme activity in control (■) in absence of pyridoxal-5′-phosphate.

shown to be composed of six identical subunits each with mol wt of 49,000 (38). Although not many higher plant glutamate dehydrogenases have been characterized with respect to mol wt and subunit composition, the native enzymes from pumpkin cotyledon (11) and pea root (35) have been shown to have mol wt of 250,000 and 200,000, respectively.

Because amino acid composition data are available for only one other plant glutamate dehydrogenase, the NADP-GDH from *C. sorokiniana* (19), the amino acid composition of the NAD-GDH from *C. sorokiniana* must be compared primarily with nonplant enzymes. The amino acid composition of the NAD-GDH from *C. sorokiniana* is distinctly different from that recently reported (19) for the NADP-GDH from this organism. The ratio of arginine to lysine residues in the NAD-GDH (*i.e.* 0.22) was much lower than that for other glutamate dehydrogenases (*i.e.* $\bar{X} = 0.85$). Moreover, when the average hydrophobicity ($H\phi_{ave}$) of the enzyme was calculated from its amino acid composition, by the method of Bigelow (8), a value of 717 cal/residue was obtained which is 339 cal/residue lower than that calculated (19) for the purified NADP-GDH from this same organism. In fact, the average hydrophobicity of most proteins, and those glutamate dehydrogenases examined, is close to 1,000 cal/residue (10). Whereas the N-terminal amino acid of the *C. sorokiniana* NAD-GDH is lysine, Gronostajski *et al.* (19) observed that α-amino group of the N-terminal amino acid of the NADP-GDH is blocked.

From kinetic studies with the NAD-GDH, it was revealed that this enzyme differs markedly from other glutamate dehydrogenases in its kinetic mechanism (Fig. 6). Other glutamate dehydrogenases evaluated, with regard to addition sequence, have been found to react by a Random (17, 30) or Ordered Ter Bi mechanism as for the safflower and soybean glutamate dehydrogenases (14, 26). However, although the kinetic mechanism of the *Chlorella* NAD-GDH appears to be different, the chemical modification studies indicate that its chemical mechanism is similar to that proposed for other glutamate dehydrogenases (40), *i.e.* formation of a Schiff base with α-ketoglutarate.

The proposed kinetic mechanism of the NAD-GDH, described by a bi uni uni uni ping-pong addition sequence, does place some constraints on a possible chemical reaction mechanism. Certain features of the *Chlorella* NAD-GDH reaction mechanism, involving the disposition of the hydride ion from NADH, are suggested by the results of the substrate exchange studies (Table V). No absorbance changes were detected in the absence of α-ketoglutarate or the enzyme, suggesting an event taking place on the enzyme surface which requires α-ketoglutarate. Moreover, because the rate at which equilibrium was approached was proportional to enzyme concentration, the equilibrium process appears to be enzyme-mediated. These data are consistent with the proposed formation of a reduced intermediate, predicted by Figure 6, requiring the presence of the enzyme, α-ketoglutarate and NADH, prior to the addition of NH_4^+.

Although the mechanism by which the reducing equivalent (*i.e.* hydride ion) is transferred from NADH is unclear, there are at least two possibilities. First, as proposed for the reaction of proline reductase (21), the hydride ion could be added directly to some component of the Schiff base intermediate. Second, as shown for the ping-pong reaction mechanism for glyceraldehyde-3-P dehydrogenase (20), the reducing equivalent could be transferred by way of a tightly bound cofactor, such as FAD^+ or NAD^+. Glutamate synthase (34) catalyzes essentially the same over-all reaction as glutamate dehydrogenase in a proposed ping-pong sequence. In the reaction of this synthase, the actual electron transfer occurs after dissociation of the $NADP^+$ from the enzyme substrate complex (9). The possibility that the NAD-GDH possesses a tightly bound cofactor, participating in hydride ion transfer, will be studied in the future in this laboratory.

Although graphically nonconvergent initial velocity patterns have indicated ping-pong addition sequences which could not be substantiated by product inhibition studies for a number of different enzymes (4–6, 16), it should be emphasized that both initial velocity and product inhibition studies are consistent with the mechanism proposed for the *Chlorella* NAD-GDH.

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