Glutamate Dehydrogenase from *Medicago sativa* L., Purification and Comparative Kinetic Studies of the Organ-Specific Multiple Forms

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NAD-specific glutamate dehydrogenase [L-glutamate: NAD^+ oxidoreductase (deaminating) EC 1.4.1.2] from *Medicago sativa* constitutes organ-specific patterns of isoenzymes. The isoenzyme-patterns of seeds (GDH-I) and roots (GDH-II) were purified 1520-fold and 92-fold, respectively. All isoenzymes of both patterns remain stable throughout the purification procedures. Isoenzyme a_7, the only isoenzyme common to both patterns was isolated from the GDH-I pattern. The three enzyme preparations were found to be identical in pH optima, substrate specificity and general kinetic properties. A comparative kinetic analysis revealed no pronounced differences between the various kinetic constants evaluated for the three enzyme preparations. Furthermore an identical order of substrate binding and product release could be established. Both initial rate measurements and product inhibition studies are consistent with an ordered ternary-binary kinetic mechanism. The results suggest that tissue-specific enzyme multiplicity of plant glutamate dehydrogenase is not related to differences in general or kinetic properties.

Introduction

The isoenzymes of NAD-dependent glutamate dehydrogenase [L-glutamate: NAD^+ oxidoreductase (deaminating) EC 1.4.1.2] have been well established for various higher plants [1–4]. Recently we have shown that GDH of *Medicago sativa* (alfalfa) constitutes organ-specific stable isoenzyme patterns [5]. One pattern (GDH-I) is present in the storage cotyledons of seeds, a second one (GDH-II) occurs in roots, whereas shoots contain a mixed pattern composed of both GDH-I and GDH-II isoenzymes. Similar tissue-specific isoenzyme patterns of GDH have been found in *Pisum sativum* (green pea) [6, 7], *Ricinus communis* (castor bean) [8], and *Lupinus albus* (white lupine) [9].

The existence of tissue-specific multiple enzyme forms may be related to cellular differentiation, subcellular compartmentation or physiological function. Because of the great differences in nitrogen metabolism between nitrogen assimilating roots and storage tissue during the mobilisation of reserve nitrogen, it has been suggested that GDH-II may be involved in anabolic processes whereas GDH-I may act predominantly catabolically [5]. Although recent research has well established that assimilation of inorganic nitrogen and formation of glutamate primarily occurs via the glutamine synthetase/glutamate synthase pathway [10, 11] it remains open whether this pathway accounts for all the glutamate produced during assimilation of inorganic nitrogen. Participation of GDH in nitrogen assimilation, at least under conditions leading to increased levels of intracellular ammonium, was recently demonstrated in experiments using the radioactive isotope ^{13}N [12]. In addition increase of GDH activity by ammonium, often accompanied by changes in the isoenzymatic patterns, has been observed in different plant tissues [13–15].

Our objectives were: (1) Purification of the two tissue specific isoenzyme patterns of GDH from seeds and roots of *M. sativa*. (2) Comparative kinetic studies of both purified patterns in order to answer the question: Does there exist a correlation between kinetic properties and tissue specificity of the isoenzyme patterns?

Materials and Methods

Plant material

Alfalfa seeds (*Medicago sativa* L.) were purchased from Samenhaus Schmitz, Bonn. Seedlings were

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Abbreviations: GDH, glutamate dehydrogenase; PVP, polyvinylpyrrolidone.

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grown on washed vermiculite in the day light without nutrient additions. After 10 days the seedlings were harvested, and the roots collected.

Chemicals

NAD^+ and NADH, grade-I were obtained from Boehringer (Mannheim) all other chemicals were of analytical grade. Polyvinylpyrolidone (PVP, unsoluble) was a gift of the BASF. DEAE-cellulose (Servacel DEAE-23-SH, 0.75–0.9 meq/g) was obtained from Serva (Heidelberg), Sepharose-6-B from Pharmacia Fine Chemicals (Freiburg).

Buffers

Tris buffers refers to 0.05 M Tris adjusted to the appropriate pH by HCl. Citrate-phosphate buffer was made by mixing 0.1 M citric acid and 0.2 M Na_2HPO_4.

Enzyme assays

GDH activity was assayed as given in [16]. The standard assay system (total volume 1.2 ml) contained the following components (final concentration): NADH reaction: 73 mM Tris (pH 8.0); 0.2 mM NADH; 16.7 mM 2-oxoglutarate; 0.125 M $(NH_4)_2SO_4$; 0.1 mM $CaCl_2$ (omitted in studies with purified enzymes). NAD^+ reaction: 79 mM Tris (pH 9.2); 2.5 mM NAD^+; 83 mM L-glutamate.

Purification of GDH-I from alfalfa seeds

Steps 1–3: Precipitation steps. Dry seeds were pulverized in a Waring Blendor; passed through a sieve (pore size 0.16 mm) and 600 g seed powder were suspended in 6 l aqua dest., containing 0.5% PVP and stirred for 1 h at room temperature. The slurry was centrifuged at 25000 × *g* for 20 min at 4 °C. All following steps were performed at 0–4 °C. The supernatant was adjusted to pH 4.0 with acetic acid agitated for 20 min and centrifuged at 25000×*g*. The pellet was resuspended in 2 l citrate-phosphate buffer pH 6.0 and stirred for 20 min; the pH was adjusted to 6.0 with cold 1 M NaOH if necessary. Insoluble protein was removed by centrifugation and an ammonium sulfate precipitate was prepared from the supernatant in the range between 35–55% saturation. The protein precipitate was dissolved in Tris buffer, pH 8.0 and dialyzed against the same buffer.

Step 4: DEAE-cellulose batch treatment. DEAE-cellulose (approx. 150 ml) equilibrated in Tris buffer pH 8.0 was added to the dialyzed enzyme solution until GDH activity in the supernatant reached zero. The loaded exchanger was poured into a Büchner-funnel and washed with 500 ml Tris buffer pH 8.0 and than with the same volume of 50-fold diluted buffer. The washed exchanger was transferred into a column (∅ 5 cm) and GDH was eluted with a 0.2 M Na-glutamate solution in Tris buffer pH 8.0. The most active fractions were pooled.

Step 5: Gel filtration on Sepharose-6-B. The pooled fractions from the DEAE-cellulose column were applied to Sepharose-6-B (2 columns 2.6 × 90 cm connected in series) and eluted with Tris buffer, pH 8.0. The most active fractions were pooled and GDH was concentrated by binding to DEAE-cellulose (20 ml) followed by elution with 0.15 NaCl or 0.2 M Na-glutamate when further purified by gel electrophoresis.

Step 6: Preparative disc-gel electrophoresis. The concentrated enzyme solution was submitted to preparative gel electrophoresis in polyacrylamide slabs (h, 15; 1, 20; d, 1.2cm) using an UltraPhor (Colora Messtechnik). Gel composition (7% acrylamide) and electrophoretic conditions were according to Maurer's system 1 a [17]. Electrophoresis was stopped when the migrating front zone labelled with bromephenol blue had reached the bottom of the gel. The gel slab was taken out and enzyme activity was localized by substrate staining [5] of small strips cut off from both sides and the middle of the slab. The zone with enzyme activity was cut out, homogenized and extracted with Tris buffer, pH 8.0. After filtration the enzyme was concentrated using a small DEAE-cellulose column as described above.

Purification of GDH-II from alfalfa roots

An acetone powder of roots from 10-days old seedlings was prepared as described [16]. The acetone powder (40 mg/ml) was suspended in Tris buffer, pH 8.0 and extracted under continuous stirring for 20 min. The suspension was centrifuged at 25000 × *g* for 20 min. The pellet was resuspended in the same volume of buffer and reextracted. The supernatants of both extracts were combined.

Further purification steps were performed as described for the purification of GDH-I including the following modifications: During acid treatment the

pH was lowered to 3.0 by slow addition of 1 N HCl; precipitation with ammonium sulfate was prepared in the range between 45–65% saturation. Batch treatment with DEAE-cellulose was omitted; the enzyme solution was directly added to a DEAE-cellulose column previously equilibrated with Tris-HCl buffer pH 8.5. Only one column was used for Sepharose-6-B gel filtration and Tris-HCl buffer pH 8.5 was used for equilibration and elution. Preparative gel electrophoresis was omitted.

Isolation of isoenzyme a_7

Purified GDH-I was added to a small DEAE-cellulose column previously equilibrated with Tris-HCl buffer pH 6.8. Isoenzyme a_7 could be eluted using the same buffer whereas the other isoenzymes remained bound.

Analytical polyacrylamide disc-gel electrophoresis

Gel electrophoresis was performed according to Maurer [17] using a separation system modified according to Jolley and Allen [18] as described previously [5]. Activity staining was performed according to [5] and protein staining as given in [19].

Results

Purification and isoenzyme patterns

The results of the purification of multiple GDH from *M. sativa* seeds (GDH-I) and roots (GDH-II) are summarized in Table I. The specific activities of the final preparations are 38 and 54 units per mg protein, respectively. The lower degree of purification obtained for GDH-II (92-fold) may be explained by the mode of the crude extract preparation. During acetone powder extraction a large portion of protein is removed on centrifugation, *i. e.* approx. 90% in pea stem preparations [21]. On the other

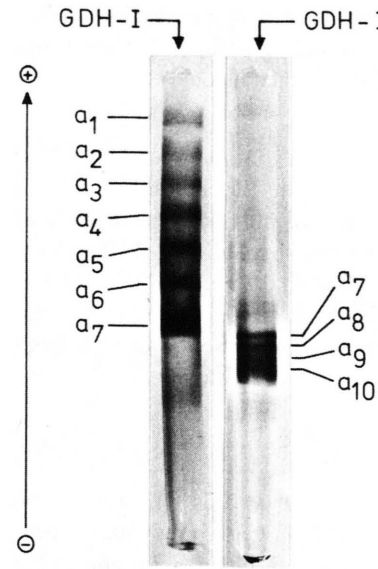


Fig. 1. Disc-electrophoretic separation of the GDH-isoenzyme patterns in crude extracts of seeds (GDH-I) and roots (GDH-II) of *M. sativa*. Separation was performed on 5.6% polyacrylamide gels; the isoenzymes were visualized by activity staining (formazan bands).

Table I. Purification of glutamate dehydrogenase from seeds (GDH-I) and roots (GDH-II) of *Medicago sativa*. 600 g seed powder (GDH-I) and 41.2 g acetone powder (GDH-II) were taken for the preparations. Protein was determined according to Lowry [20] using human serum albumin as standard; activity measurements were performed under standard assay conditions (NADH-reaction).

Purification step	Total activity (units [a])		Specific activity (units/mg protein)		Yield (%)		Purification	
	GDH-I	GDH-II	GDH-I	GDH-II	GDH-I	GDH-II	GDH-I	GDH-II
1. Crude extract	1540	429	0.025	0.59	100	100	1	1
2. pH treatment	1648	268	0.075	4.4	107	62	3	8
3. Ammonium sulfate precipitate	1269	203	0.51	5.1	82	47	20	9
4. DEAE-cellulose	967	101	1.7	8.1	63	24	68	14
5. Sepharose-6-B after concentration	549	70	8	54	36	16	320	92
6. Electrophoresis after concentration	352		38		23		1520	

[a] One unit catalyzes the oxidation of 1 μmol NADH per min.

hand the high degree of purification of GDH-I (1520-fold) reflects the removal of large amounts of seed storage protein during the purification procedure.

The electrophoretic isoenzyme patterns of crude GDH-I and GDH-II are shown in Fig. 1. Both patterns remain stable throughout the purification procedure. The final preparations of GDH-I were found to be homogenous as judged from gel electrophoresis whereas the GDH-I preparation still shows some contaminating protein. Isoenzyme a_7 the only isoenzyme common to both patterns was separated from the other isoenzymes of the GDH-I pattern in a yield of about 18% of total GDH activity by means of DEAE-cellulose chromatography.

General properties

Under standard assay conditions using 0.05 M Tris buffers the pH optima for GDH-I and GDH-II were 8.0–8.25 (NADH-dependent reaction) and 9.2 (NAD^+-dependent reaction). However it was found that the optimal pH for the NAD^+ reaction depends on the glutamate concentration. Values between pH 8.5–9.5 were determined by varying the glutamate concentration between 0.02–0.22 M.

Since several plant GDHs are activated by Ca^{2+} and other divalent metal ions [16, 21–23], the effects of metal ions on enzyme activity in the various stages of purification were controlled. Partially purified enzyme preparations at least of GDH-I showed some (up to 3-fold) activation by Ca^{2+}. However, the purified enzyme partially inactivated by pretreatment with EDTA [16] could not be reactivated by addition of Ca^{2+}. Thus studies with purified enzymes were performed in the absence of Ca^{2+}.

With the three purified enzyme preparations linear Lineweaver-Burk plots were obtained with all substrates over the concentration range tested. Ammonium was usually added as ammonium sulfate. Sigmoidal kinetics as reported by Pahlich [24] were not observed for 2-oxoglutarate when ammonium sulfate was replaced by ammonium chloride. However, a pronounced competitive behaviour was found to exist between 2-oxoglutarate and SO_4^{2-} or Cl^- (Fig. 2). With 0.1 M SO_4^{2-} an apparent K_M for 2-oxoglutarate of 5.3 mM can be calculated which is approx. 4-times higher than an apparent K_M of 1.3 mM evaluated for a fictive anion concentration of zero.

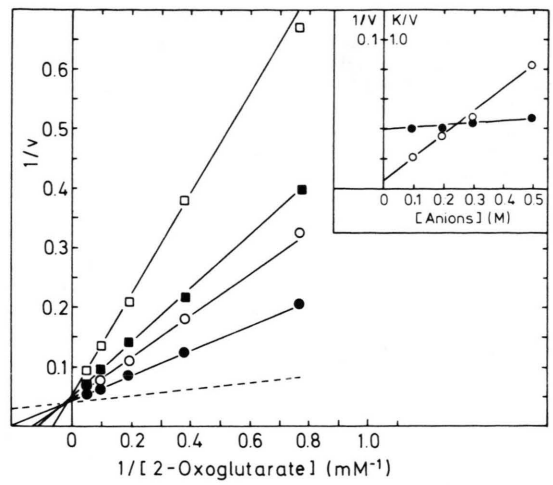


Fig. 2. Double reciprocal plots of anion inhibition of the reductive aminating reaction catalyzed by GDH-I. NADH and NH_4^+-concentrations were 0.2 mM and 0.2 M, respectively. 2-Oxoglutarate was varied against several fixed anion concentrations: (●) 0.1 M $(NH_4)_2SO_4$; (○) 0.2 M NH_4Cl; (■) 0.2 M NH_4Cl + 0.1 M Na_2SO_4; (□) 0.1 M $(NH_4)_2SO_4$ + 0.4 M NaCl; (- - -) fictive anion concentration zero (calculated from secondary plot). Inset: Secondary plot, (○) slopes (K/V) and (●) intercepts (1/V) against total anion concentration.

Ammonium binding is not influenced by Tris concentrations up to 0.2 M.

Preliminary substrate kinetic studies revealed that the apparent Michaelis constants of ammonium depend upon the concentrations of 2-oxoglutarate and NAD and vice versa. The apparent K_M of ammonium is small at low levels of NADH and increases with rising levels of NADH (Table II).

The substrate specificity of purified GDH-I and GDH-II is shown in Table III. Comparison of both enzyme preparations displays no differences. NADPH and $NADP^+$ can be used to some extent instead of NADH and NAD^+, respectively, which is already known from GDH of other plant sources [3, 16, 25,

Table II. Dependency of the apparent K_M-constants of ammonium on the concentration of NADH (GDH-I). Assays were performed at fixed levels of 2-oxoglutarate (3 mM) and varying fixed levels of NADH.

NADH in the assay [μM]	Apparent K_M of NH_4^+ [mM]
5	3.6
10	6.8
50	22.0
100	30.0

Table III. Substrate specificity of GDH-I and GDH-II. The complete assay system containes: NADH reaction, 0.5 mM NADH; 16.6 mM 2-oxoglutarate; 0.2 M $(NH_4)_2SO_4$. NAD^+ reaction, 5 mM NAD^+; 0,25 M L-glutamate (for comparison of NAD^+ with $NADP^+$) or 50 mM L-glutamate (for comparison with alternative amino acids). Concentrations of alternative substrates: NADPH, 0.5 mM; $NADP^+$, 5 mM; 2-oxoacids, 16.6 mM; L- or D-amino acids, 50 mM; D,L-amino acids, 100 mM.

	GDH activity (per cent)	
	GDH-I	GDH-II
Complete assay system	100	100
NADH omitted + NADPH	5	5
NAD^+ omitted + $NADP^+$	8	8
2-Oxoglutarate omitted		
+ Oxaloacetate	0	0
+ Pyruvate	< 1	0
+ 2-Oxobutyrate	< 1	0
+ 2-Oxovalerate	2.2	2.5
+ 2-Oxocaproate	< 1	0
L-Glutamate omitted		
+ L-Asparate	1.4	< 1
+ D-Glutamate	2.3	1.2
+ L-Alanine	1.2	< 1
+ D,L-Norvaline	3.3	2.9
+ D,L-Glutamate	N. D.	39

N. D. = not determined.

26]. The small degree of activity with 2-oxovalerate, L-alanine and D, L-norvaline is similar to that shown for the animal enzyme. D-Glutamate which acts to some extent as amino group donator is a potent inhibitor of the oxidative reaction. Furthermore neither glutamine nor asparagine or methylamine could replace NH_4^+ as a substrate.

Initial velocity measurements

NADH reaction: The general description of a three substrate reaction which yields linear Lineweaver-Burk plots with respect to all substrates with any fixed concentration of the other two substrates can be formulated by Eqn (1) [27] modified according to Laidler and Bunting [28]:

$$\frac{1}{v} = \frac{1}{V} + \frac{K_M^A}{V[A]} + \frac{K_M^B}{V[B]} + \frac{K_M^C}{V[C]} + \frac{K_M^A K_M^B}{V[A][B]} + \frac{K_S^A K_M^C}{V[A][C]} + \frac{K_S^B K_M^C}{V[B][C]} + \frac{K_S^A K_S^B K_M^C}{V[A][B][C]}$$

Eqn (1). K_M^A, K_M^B, K_M^C are the Michaelis constant for the substrates A, B, C; K_S^A and K_S^B are the dissociation constants of the enzyme-substrate-complex EA and EAB, respectively; v = initial reaction velocity; V = maximal reaction velocity.

To determine the kinetic parameters given in Eqn (1) initial velocity measurements were made with four concentrations of each substrate (NADH, 2-oxoglutarate, NH_4^+) in all combinations, and evaluated graphically as recommended by Dalziel [28]. Fig. 3 shows the various plots for a kinetic series in which A (NADH) was used as the primary variable. For control the same plots (not shown) were made with B (2-oxoglutarate) and C (NH_4^+) as the primary variable, yielding altogether six estimates for each parameter.

The kinetic parameters are nearly identical for GDH-I and GDH-II (Table IV). As shown in the table the binary term $K_S^A K_M^C$ is zero, which is a strong indication for an ordered sequential mecha-

Table IV. Kinetic constants for the reaction catalyzed by the multiple forms of glutamate dehydrogenase from *Medicago sativa*. A = NADH; B = 2-Oxoglutarate; C = NH_4^+; D = L-Glutamate; E = NAD^+. K_M = Michaelis constant; K_S = Dissoziation constant.

Enzyme preparation	Reductive amination reaction [a]									Oxidative deamination reaction [b]	
	K_M^A [μM]	K_M^B [mM]	K_M^C [mM]	$K_S^A K_M^B$ [μM²]	K_S^A [μM]	$K_S^A K_M^C$ [mM²]	$K_S^B K_M^C$ [mM²]	K_S^B [mM]	$K_S^A K_S^B K_M^C$ [μM³]	K_M^D [mM]	K_M^E [μM]
GDH-I	72.1 ±4.8	2.6 ±0.26	70.1 ±3.4	0.162 ±0.019	64.6 ±7.1	0	0.133 ±0.019	1.84 ±0.21	0	53.1 ±3.3	349 ±12
GDH-II	77.5 ±3.7	3.42 ±0.16	83.4 ±3.1	0.259 ±0.024	77.1 ±6.9	0	0.167 ±0.013	2.07 ±0.21	0	28.4 ±0.7	377 ±22
Isoenzyme a_7	111 ±14	4.01 ±0.75	139 ±11	0.82 ±0.15	187 ±24	0	0.377 ±0.068	3.9 ±0.4	0	102.9 ±5.9	353 ±43

Values are: Mean ± S.E.M.

[a] Mean values of the kinetic parameters are obtained from two different experiments according to [27] yielding alltogether 12 estimates of each parameter.

[b] Mean values obtained from three different experiments yielding alltogether 6 estimates for each parameter.

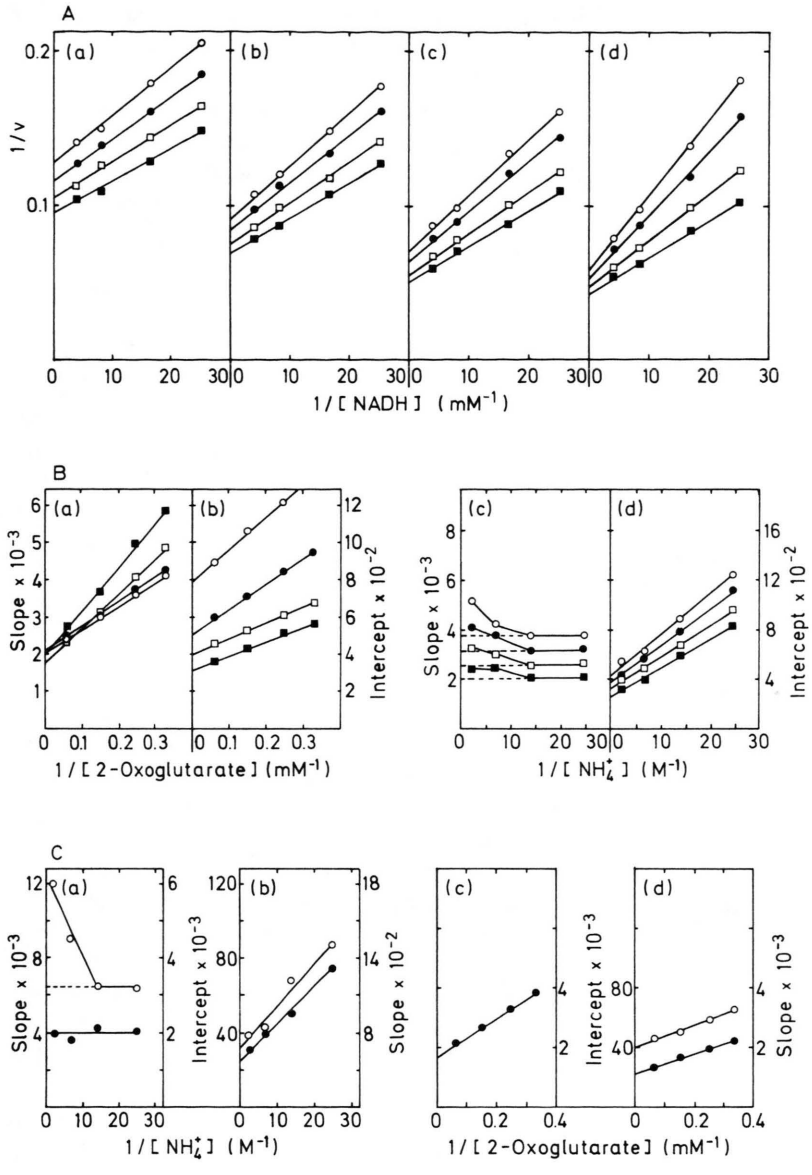


Fig. 3. Initial velocity measurements of the reductive amination reaction catalyzed by GDH-I. A, Primary Lineweaver-Burk plots with the concentrations of NADH as variable at four fixed levels of NH_4^+: (a) 0.04 M; (b) 0.07 M; (c) 0.14 M; (d) 0.4 M. 2-Oxoglutarate concentrations in each graph are: (○) 3 mM; (●) 4 mM; (□) 6.5 mM; (■) 15 mM. B, Secondary plots of (a) slopes and (b) intercepts from Figs. A against reciprocal concentrations of 2-oxoglutarate at four fixed levels of NH_4^+: (○) 0.04 M; (●) 0.07 M; (□) 0.14 M; (■) 0.4 M. Secondary plots of (c) slopes and (d) intercepts from Figs. A against reciprocal concentrations of NH_4^+ at four fixed levels of 2-oxoglutarate: (○) 3 mM; (●) 4 mM; (□) 6.5 mM; (■) 15 mM. C, Tertiary plots. (○) Slopes and (●) intercepts of corresponding Figs. B (a) to B (d) against reciprocal concentrations of NH_4^+ and 2-oxoglutarate, respectively.

nism in which B (2-oxoglutarate) adds as the second substrate [27]. The determination of the term $K_S^A K_S^B K_M^C$ was complicated because of the ammonium sulfate inhibition (see below). It was evaluated at infinite concentrations of A, B or C. At infinite concentrations of each of three substrates the ternary term reduces to zero [39]. This is obvious for infinite concentrations of B where the rate equation reduces to that of a pingpong mechanism. The ternary term estimated from the tertiary plots was in fact zero (Table IV).

Secondary and tertiary plots of the slopes against the reciprocal concentrations of NH_4^+ were upward curved in the range of high NH_4^+ concentrations (see Fig. 3Bc and Ca). Since only the curve with the slope is affected a competitive substrate inhibition is probable [30], which reflects the competitive inhibition of 2-oxoglutarate binding by SO_4^{2-}, the counterpart anion of NH_4^+.

NAD^+ reaction: Initial velocity measurements were made at pH 9.2 with five concentrations of NAD^+ and L-glutamate, respectively. An intersecting

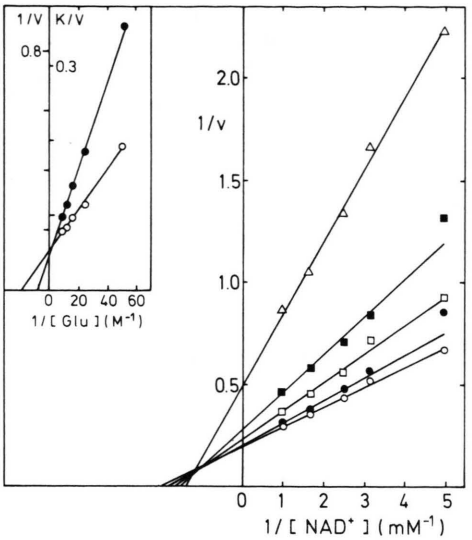


Fig. 4. Initial velocity measurements of the oxidative deaminating reaction catalyzed by GDH-I. A, Primary Lineweaver-Burk plots with the concentrations of NAD^+ as variable at five fixed levels of L-glutamate: (○) 20 mM; (●) 40 mM; (□) 60 nM; (■) 80 nM; (△) 100 mM. Inset: Secondary plots of (●) slopes (K/V) and (○) intercepts (1/V) of primary plots against reciprocal concentrations of L-glutamate.

pattern with linear primary and secondary plots was obtained (Fig. 4). The K_M values evaluated for the three enzyme preparations are given in Table IV. The mean K_M values obtained for NAD^+ are identical for the three enzyme-systems, whereas the respective values for L-glutamate differ by a factor of 2 to 4.

Product inhibition studies

The forgoing initial velocity measurements suggest an ordered sequential mechanism of substrate binding in which NADH binds as the first substrate followed by 2-oxoglutarate (second substrate) and NH_4^+ (third substrate).

To obtain unequivocal evidence product inhibition studies were required. Since the initial velocity studies revealed identical kinetic patterns for the three enzyme systems product inhibition studies were only performed with isoenzyme a_7.

the results are shown in Fig. 5. All primary and secondary plots were linear. The observed inhibition patterns are summarized in Table V in comparison to the patterns predicted for an ordered sequential mechanism according to Cleland [29–32]. Since in our experiments the nonvaried substrates were present at well saturating levels two patterns turned out to be uncompetitive rather than noncompetitive which still fits the given predictions. However, there is one clear deviation from the predicted pattern: L-Glutamate served as a competitive inhibitor of 2-oxoglutarate when it should be an uncompetitive one.

Discussion

It has been suggested that the isoenzymes composing the organ-specific GDH isoenzyme patterns of *M. sativa* [5] and *Pisum sativum* [6, 11] differ in charge rather than in size. This view which is supported by the present study could be proved recently by sedimentation equilibrium measurements and sodium dodecyl sulfate electrophoresis of homogenously pure multiple GDH isolated from pea seeds [33]. Thus the term "charge isoenzymes" may be applied to characterize the individual multiple enzyme forms composing the organ specific patterns of GDH from *P. sativum* and *M. sativa*.

Table V. Predicted product inhibition patterns of an ordered sequential mechanism with NADH as the first, 2-oxoglutarate the second and NH_4^+ the third substrate to bind, and observed patterns with glutamate dehydrogenase isoenzyme a_7.

Varied substrate	Inhibitor			
	NAD^+		L-Glutamate	
	predicted	observed	predicted	observed
NADH	C	C	NC [a]	UC
2-Oxoglutarate	NC [a]	UC	NC [a]	C
Ammonium	NC [a]	NC	NC	NC

C = competitive, UC = uncompetitive, NC = noncompetitive.
[a] The inhibition pattern may change from NC to UC if the non-varied substrates are present in saturating concentrations.

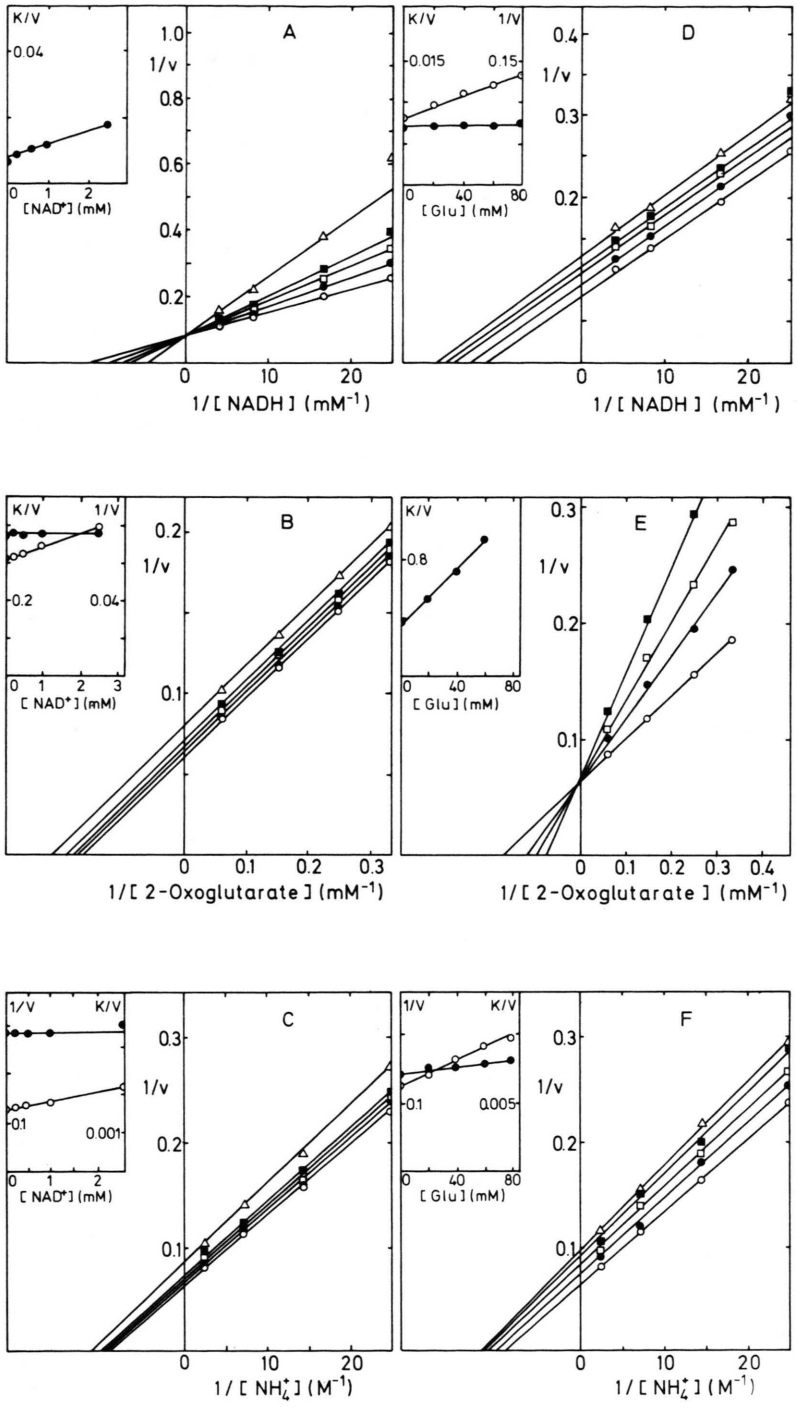


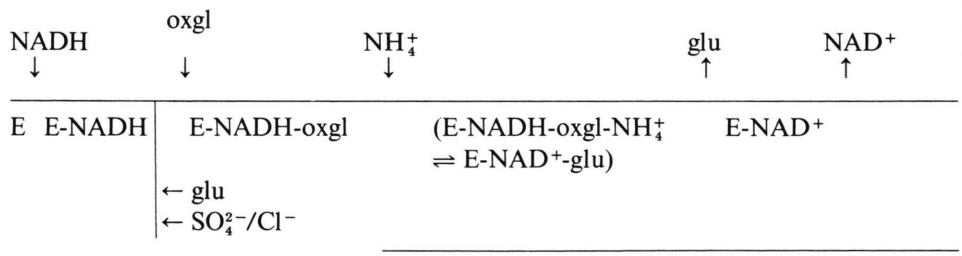
Fig. 5. Double reciprocal plots of product inhibition of the reductive amination reaction catalyzed by GDH-I isoenzyme a_7. (A) NADH, (B) 2-oxoglutarate, (C) NH_4^+ as varied substrate against several fixed concentrations of NAD^+: (○) no inhibitor; (●) 0.25 mM; (□) 0.5 mM; (■) 1 mM; (△) 2.5 mM. (D) NADH, (E) 2-oxoglutarate, (F) NH_4^+ as varied substrate against several fixed concentrations of L-glutamate: (○) no inhibitor; (●) 0.02 M; (□) 0.04 M; (■) 0.06 M; (△) 0.08 M. The respective non varied substrates were held at fixed saturating levels: NADH, 0.5 mM; 2-oxoglutarate, 16.7 mM; NH_4^+, 0.4 M. Inset: replots of (●) slopes (K/V) and (○) intercepts (1/V) against inhibitor concentration.

A comparison of the true K_M values evaluated for GDH-I, and GDH-II revealed that the K_M values of at least four substrates (NADH, NAD^+, 2-oxoglutarate, NH_4^+) have the same order of magnitude. Isoenzyme a_7 which is common to both isoenzyme patterns has a slightly higher K_M for NADH, 2-oxoglutarate and NH_4^+ than GDH-I or GDH-II and a K_M for glutamate which is about 2-fold and 4-fold higher than that of GDH-I and GDH-II, respectively. It appears doubtful whether these differences are high enough as to be related to functional differences of the two tissue specific patterns. In addition no differences could be detected in pH dependency and substrate specificity.

The kinetic patterns of initial velocity studies show that the binding of substrates is identical for both isoenzyme patterns and isoenzyme a_7. The kinetic data suggest an ordered Ter Bi mechanism of substrate binding. This mechanism is consistent with various single kinetic data presented for other plant NAD-specific GDHs [34–36], but is in contrast to a partially random mechanism recently proposed for the pea enzyme by Garland and Dennis [21]. The authors observed a random binding of NH_4^+ and 2-oxoglutarate. Our data show that, as requested for an ordered sequential mechanism, the reversible sequence between NADH and NH_4^+ is broken, when the concentration of oxoglutarate (second substrate) is infinite.

An ordered sequential mechanism is further supported by the observed product inhibition pattern which coincides with the predicted pattern except the deviation that glutamate acts as a competitive inhibitor of 2-oxoglutarate. Since various anions were shown to be competitive inhibitors of 2-oxoglutarate one explanation would be that this type of inhibition is also caused by glutamate which is present in the assay in much higher concentrations than 2-oxoglutarate. Furthermore glutamate and 2-oxoglutarate must share a common binding site and if 2-oxoglutarate can form a productive ternary complex with enzyme-NADH the formation of an analogous dead-end complex with glutamate is not surprising [33].

The following kinetic mechanism which summarizes the various kinetic data is suggested:



The comparison of the two tissue specific isoenzyme patterns of GDH from *M. sativa* clearly shows that both patterns do not differ in their kinetics and general properties, which has been supposed for various multiple plant GDHs in respect to tissue specific functional differences [5, 7–9]. Since in *P. sativum* the isoenzymes of both patterns were shown to be located exclusively within the mitochondria, association of the multiple forms with different intracellular compartiments can be excluded, too [38]. Furthermore in *M. sativa* the isoenzyme patterns are not influenced quantitatively or qualitatively by exogenous NH_4^+ as reported for several plant enzymes [13–15, 37, 38]. Thus the question still remains whether tissue specific isoenzyme patterns account for physiological differences or simply reflect cellular differentiation.

It is clear that the true K_M constants evaluated in these studies do not represent the apparent constants one would obtain working with the substrate concentrations and the pH of the living cell. The apparent K_M values are known to vary with pH [41] and as shown above strongly depend on the concentrations of the other substrates. The apparent K_M for NH_4^+ of about 4 mM determined in presence of low concentrations of NADH, which is nearly 20-fold lower than the "true K_M" for NH_4^+ evaluated for an infinite concentration of NADH, is rather well in correspondence to the halfe saturation values for NH_4^+ obtained with isolated pea mitochondria [38].

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