Plant NAD-Dependent Glutamate Dehydrogenase. Purification, Molecular Properties and Metal Ion Activation of the Enzymes from *Lemna minor* and *Pisum sativum*

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Glutamate dehydrogenase (L-glutamate: NAD^+ oxidoreductase (deaminating) EC 1.4.1.2) has been purified to homogeneity from *Lemna minor* and seeds of *Pisum sativum.* As established by polyacrylamide gel electrophoresis the *Pisum*-enzyme constitutes a multiple pattern of seven charge isoenzymes whereas the *Lemna* enzyme shows one single protein band. Molecular weights of 230 000 were calculated for both enzymes by sedimentation equilibrium measurements (*Pisum*-enzyme) and comparative gel filtration (*Lemna*-enzyme). Sodium dodecyl sulfate gel electrophoresis and electron microscopic observations revealed that both enzymes are composed of four identical subunits (molecular weight 58 500) arranged in a tetraedric structure. The amino acid compositions of both enzymes are similar to those of various hexameric glutamate dehydrogenases. The N-terminal amino acid of the *Pisum*-enzyme is alanine. Both enzymes require Ca^{2+} for maximal catalytic activity. For the *Lemna*-enzyme the $K_{0.5}$ values for Ca^{2+} are 22 μM (NADH-dependent reaction) and 4 μM (NAD^+-dependent reaction), respectively. Ca^{2+} which to some extent can be replaced by Zn^{2+} does not affect the enzyme aggregation but seems to govern a reversible equilibrium between catalytically active and inactive enzyme forms.

Introduction

Mitochondrial glutamate dehydrogenase [L-glutamate: NAD^+ oxidoreductase (deaminating), EC 1.4.1.2] has been more or less highly purified from various plants [1–5]. Although the enzyme has been extensively studied its molecular properties are still unknown. Definite differences exist between plant glutamate dehydrogenase and the corresponding enzymes from vertebrates and microorganisms, particularly concerning regulatory properties. Two features of plant glutamate dehydrogenase require attention and clarification: (a) The enzyme multiplicity which has been established for a great number of plants [see 6, 7]; the multiple enzyme forms of plant glutamate dehydrogenase can constitute tissue specific patterns which may be changed during development or experimental manipulation [8–13]. (b) The sensitive activation of the aminating and deaminating reactions by divalent metal ions [2, 14 to 16]. For detailed studies of both features pure and homogeneous enzymes are needed.

In this communication we describe the purification to homogeneity and the molecular properties of two glutamate dehydrogenases, (a) the multiple enzyme from pea seeds, (b) the uniform enzyme from *Lemna minor* which previously has been found suitable for studies of the metal ion activation.

Reprint requests to Professor Dr. T. Hartmann.

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Materials and Methods

Materials

Cultivation of *Lemna minor* and preparation of acetone dry powders were as described in ref. [16]. Seeds of *Pisum sativum* "Presto" were a gift of van Waveren-Pflanzenzucht (Rosdorf).

Chemicals

Pyridine nucleotides (NAD^+, NADH, $NADP^+$, NADPH) were from Boehringer (Mannheim); grade-I was used for enzymatic activity assays, grade-II for affinity elution and in electrophoretic studies. Sepharose 4 B and 6 B, and Sephadex G-200 were purchased from Pharmacia Fine Chemicals (Frankfurt). DEAE-cellulose (Servacel DEAE-23-SH, 0.93 meq/g) was obtained from Serva (Heidelberg). All other chemicals were of analytical grade.

Buffers

Tris buffers refer to Tris molarities as indicated, adjusted to the appropriate pH by HCl. Buffer A:

0.05 M Tris, pH 7.5. Citrate-phosphate buffer was made by mixing 0.1 M citric acid and 0.2 M Na_2HPO_4.

Gel electrophoresis

Polyacrylamide disc-gel electrophoresis was performed according to Maurer [17] using the separation system 1 a (*Lemna*-enzyme) and a separation system modified according to ref. [18] as described previously [9]. Activity staining was performed as described in ref. [9] and protein staining with Coomassie Brilliant Blue R as given by ref. [19]. Sodium dodecyl sulfate gel electrophoresis was carried out in tubes or slabs according to ref. [20]. The protein was stained with Coomassie blue.

Ultracentrifugation studies

Sedimentation equilibrium measurements were carried out in a Beckman ultracentrifuge model E equipped with an ultraviolet scanner at 5 °C. Runs were performed at 5600 and 11000 rev./min using the meniscus-depletion technique described by Yphantis [21]. Samples with protein concentrations of 0.3 to 0.4 mg/ml in buffer A were used corresponding to a extinction (E_{280}) of 0.5 – 0.7. The molecular weight was obtained from a plot of ln c versus r^2 according to ref. [22]. Sedimentation velocity measurements were conducted at 60000 rev./min at 20 °C.

Electron microscopy

The purified enzymes (0.1 – 0.15 mg protein/ml buffer A) were negatively stained using the procedure given by ref. [23]. Electron micrographs were taken with a Philips EM 301. Primary magnification: 46500 ×.

Amino acid analysis

The amino acid analysis and the determination of the N-terminal amino acids were performed by Dr. B. Wittmann-Liebold (Max-Planck-Institut für Molekulare Genetik, Berlin-Dahlem). Sample preparation, apparatus and procedures were as given in ref. [24].

Enzyme assay

Glutamate dehydrogenase activity was assayed photometrically at 366 nm as given in ref. [15] using an Eppendorf photometer equipped with recorder and a thermostated cell holder at 25 °C. The enzymatic assays (total volume 1.2 ml) contained the following components (final concentrations): *Lemna*-enzyme, reductive amination: 75 mM Tris, pH 8.0; 0.21 mM NADH; 16.7 mM 2-oxoglutarate; 167 mM NH_4Cl; 1 mM $CaCl_2$. Oxidative desamination: 83 mM Tris, pH 8.7; 0.83 mM NAD^+; 16.7 mM L-glutamate; 1 mM $CaCl_2$. *Pisum*-enzyme, reductive amination: 75 mM Tris, pH 7.8; 0.21 mM NADH; 16.7 mM 2-oxoglutarate; 125 mM $(NH_4)_2SO_4$; 0.1 mM $CaCl_2$. Oxidative desamination: 83 mM Tris, pH 8.5; 2.5 mM NAD^+; 83 mM L-glutamate.

Metal ion activation was measured using purified enzyme preparations previously dialyzed for 24 h against buffer A containing 60 µmol/l Ethyleneglycol-bis-(β-aminoethylether)-N,N′-tetraacetic acid (EGTA). Ca^{2+} and Zn^{2+} were added as chlorides.

Protein determinations

Protein was estimated according to ref. [25]. In purified preparations the protein content was additionally evaluated from the absorbance at 280 nm.

Purification of glutamate dehydrogenase from pea seeds

Crude extracts: Dry pea seeds were pulverized in a Waring Blendor and passed through a sieve (pore size 0.16 mm). Aliquots of 220 g seed powder were suspended in 600 ml buffer A containing 5 drops Triton-X-100, stirred for 20 min and centrifuged at 25000 × g for 10 min. The pellet was reextracted in the same volume of buffer A (minus Triton). The supernatants of the two extractions were combined.

Acid precipitation: The crude extract was adjusted to pH 4.0 by slow addition of 1 M HCl, agitated for 15 min and centrifuged at 25000 × g for 10 min. The pellet was carefully resuspended in 600 ml citrate-phosphate buffer, pH 6.0, stirred for 90 min, and insoluble materials were removed by centrifugation at 25000 × g for 10 min.

Blue-dextran Sepharose 4 B chromatography: The affinity gel was prepared from previously cyanogen bromide activated Sepharose 4 B [26] according to Ryan and Vestling [27]. Two blue-dextran Sepharose columns (2.7 × 10 cm; 40 ml gel) were prepared and 300 ml supernatant of the acid precipitation step were applied to each column. The columns were washed with buffer A until the effluent was free of

protein. To remove further contaminating protein each column was eluted with 10 ml 1 mM ATP in buffer A followed by 10 ml 1 mM NAD^+ in buffer A. Finally glutamate dehydrogenase was eluted with 20 ml buffer A containing 1 mmol/l NADH.

Gel filtration: The enzyme solution was applied to a Sepharose 6 B column (2.6 × 85 cm) and eluted with buffer A.

DEAE-cellulose chromatography: The enzymatically active fractions of the gel filtration were combined and applied to a DEAE-cellulose column (1.2 × 7 cm; 2.5 ml) previously equilibrated with buffer A, washed with 50 ml of the same buffer followed by 400 ml buffer A, pH 8.0. The enzyme was eluted with 10 ml 0.15 M NaCl in buffer A.

Dialysis and concentration: The final enzyme solution was dialyzed for 16 – 20 h against buffer A. In some experiments dialysis was preceded by a concentration step: ammonium sulfate precipitation at 90 – 95% saturation.

The dialyzed enzyme could be stored at – 18 °C without loss of activity for at least 4 weeks.

Purification of glutamate dehydrogenase from L. minor

Crude extracts and ammonium sulfate precipitation: 40 g acetone powder were suspended in 600 ml buffer A, stirred for 30 min at room temperature and centrifuged at 25000 × *g* for 15 min. The pellet was reextracted with buffer A. An ammonium sulfate precipitate was prepared from the combined supernatants in the range between 45 and 70% saturation. The precipitate was redissolved in buffer A and desalted by Sephadex G-50 gel filtration.

Heat precipitation: The Sephadex eluate was kept at 60 °C for 10 min, cooled in an ice bath, centrifuged at 25000 × *g* for 10 min and the heat precipitation was repeated for 5 min at 60 °C. The resulting supernatant was applied to a blue dextran column.

Affinity and gel chromatography: The procedure for blue-dextran Sepharose 4 B affinity chromatography followed by Sepharose 6 B gel filtration was the same as given above for the pea enzyme. Only one column was used for affinity chromatography.

DEAE-cellulose chromatography: The enzymatically active fractions of the Sepharose eluate were concentrated using an Amicon ultra-filter-cell (Diaflow UM 20) and the buffer was changed to 0.02 M Tris, pH 8.2. The concentrated enzyme solution (15 to 20 ml) was bound to a DEAE-cellulose column (1.2 × 7 cm; 5 ml) equilibrated with 0.02 Tris buffer, pH 8.2. The column was washed with 100 ml equilibration buffer and glutamate dehydrogenase was eluted with 10 ml 0.05 M Tris buffer, pH 7.5 containing 20 mmol/l $CaCl_2$.

The enzyme could be stored at – 18 °C without loss of activity for at least 4 weeks.

Results and Discussion

Purification

The results of the purification of glutamate dehydrogenase from seeds of *P. sativum* and axenically cultured *L. minor* are summarized in Table I and Table II, respectively. The final preparations of the enzymes from both sources have specific activities of about 400 – 500 units/mg protein.

The acid precipitation step [1] was found useful for the prepurification of the seed enzyme, as large amounts of proteinous and non-proteinous seed-storage materials were removed. By contrast this method gave poor results with the *Lemna*-enzyme which in turn could be sufficiently pre purified by fractionated ammonium sulfate precipitation followed by a heat precipitation step. The selective purification of both enzymes by pseudoaffinity chromatography on blue-dextran Sepharose 4 B was further improved by washing with 1 mM ATP and NAD^+. By this procedure critical dextran-blue bound proteins could be removed which otherwise contaminated the final preparations. Glutamate dehydrogenase activity was not solubilized during this procedure. The final purification was achieved by ion exchange chromatography. The different procedures established for the two enzymes are not interchangeable indicating considerable charge differences between the two species of glutamate dehydrogenase, which also can be seen from the different electrophoretic behaviour.

Homogeneity and multiple enzyme forms

The results of the electrophoretic studies are shown in Fig. 1. Polyacrylamide gel electrophoresis of the purified *Lemna*-enzyme indicates a single protein band containing all the glutamate dehydrogenase

Table I. Purification of glutamate dehydrogenase from seeds of *Pisum sativum.*

Purification step	Total activity [U]*	Specific activity [U/mg protein]	Purification (fold)	Recovery [%]
Crude extract	720	0.039	1	100
Acid precipitation	583	0.21	5.4	81
Blue-dextran-Sepharose 4B	425	45.3	1 167	59
Sepharose 6 B	396	92.0	2 371	55
DEAE-cellulose	274	401.0	10 335	38

* One unit (U) is defined as the enzyme necessary to oxidize 1 µmol of NADH/min. Preparation was started from 220 g seed powder.

Table II. Purification of glutamate dehydrogenase from *Lemna minor.*

Purification step	Total activity [U]*	Specific activity [U/mg protein]	Purification (fold)	Recovery [%]
Cruce extract	661	0.45	1	72
Ammonium sulfate precipitate, 45 – 70%	918	1.8	4	100
Heat precipitation	725	3.8	8.4	79
Blue-dextran-Sepharose 4B	588	136	302	64
Sepharose 6B	525	252	560	57
DEAE-cellulose	405	540	1200	44

* One unit (U) is defined as the enzyme necessary to oxidize 1 µmol NADH/min. Preparation was started from 40 g acetone powder.

activity. Gel electrophoresis of the *Pisum*-enzyme shows seven protein bands coincident with glutamate dehydrogenase activity. The results of sodium dodecyl sulfate electrophoresis indicate single bands of identical mobility for both enzyme preparations. The purity of the enzyme preparations was further proved by sedimentation equilibrium studies in the ultracentrifuge and by electron microscopic observation.

The multiple forms observed for the purified pea enzyme are identical with those detected in crude extracts of pea seeds by activity staining. The homogenous behaviour of the purified preparation in sedimentation velocity runs and sodium dodecyl sulfate gel electrophoresis confirms earlier results with crude enzyme preparations suggesting that the multiple forms of the enzyme differ in charge rather than in size [8, 10]. The intensity of the individual bands was unequal when stained for protein or activity. The major band had the least mobility toward the anode (Fig. 2).

Molecular weight and subunit composition

The molecular weight of the native pea enzyme was determined by sedimentation-equilibrium (Fig. 3). At protein concentrations of 0.3 – 0.4 mg per ml identical molecular weights of 230 000 ± 1 000 were obtained on the basis of a partial specific volume of $\bar{v} = 0.746$ calculated from the amino acid composition (minus tryptophan and cysteine). The molecular weight of the *Lemna*-enzyme which could not be obtained in sufficient quantities for a detailed sedimentation-equilibrium analysis was determined using gel filtration on Sephadex G-200. Co-chromatography with the pea enzyme gave only one symmetrical peak with glutamate dehydrogenase activity. Gel electrophoresis of the enzymatically active fraction revealed no differences in the distribution pattern of the two enzymes. Thus identical molecular weights for both enzymes can be assumed.

The subunit molecular weight was determined by sodium dodecyl sulfate gel electrophoresis (Fig. 1). Identical values of 58 500 ± 1 500 daltons were esti-

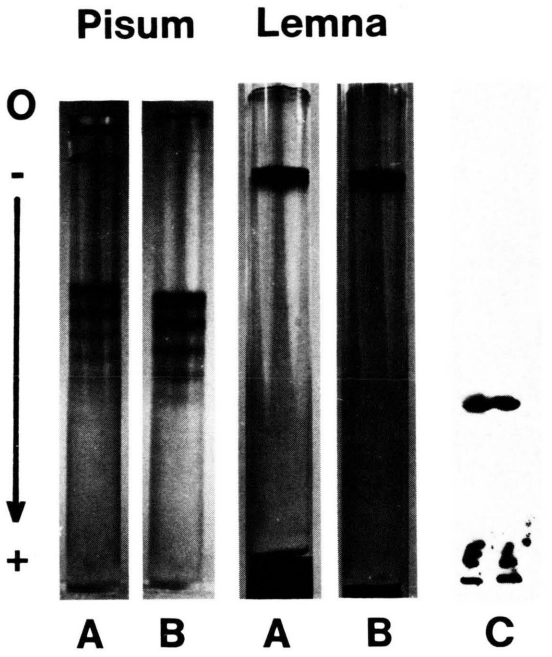

Fig. 1. Polyacrylamide disc-gel electrophoresis of purified glutamate dehydrogenase from *P. sativum* (5.6% gels) and *L. minor* (7% gels). A = Activity staining (formazan bands). B = protein staining (Coomassie-Blue bands). C = Sodium dodecyl sulfate gel electrophoresis on 8% polyacrylamide slab-gel; E = *Pisum*-enzyme; L = *Lemna*-enzyme.

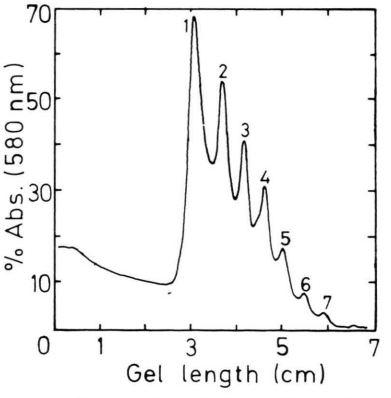

Fig. 2. Separation of the multiple forms of purified glutamate dehydrogenase from *P. sativum* by polyacrylamide gel electrophoresis. Scanner tracing of gels at 580 nm after activity staining. (Anode left.)

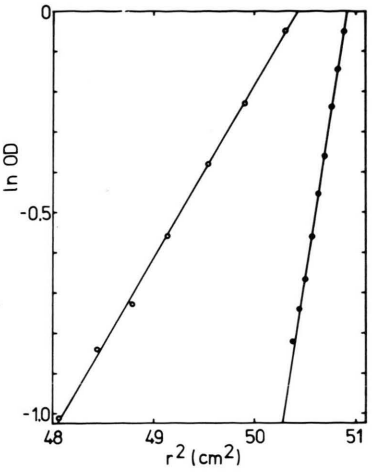

Fig. 3. Sedimentation equilibrium of native glutamate dehydrogenase from *P. sativum* in buffer A. Centrifugation was performed at 5 °C and rotor speeds of 5600 rpm (open circles) and 11000 rpm (closed circles).

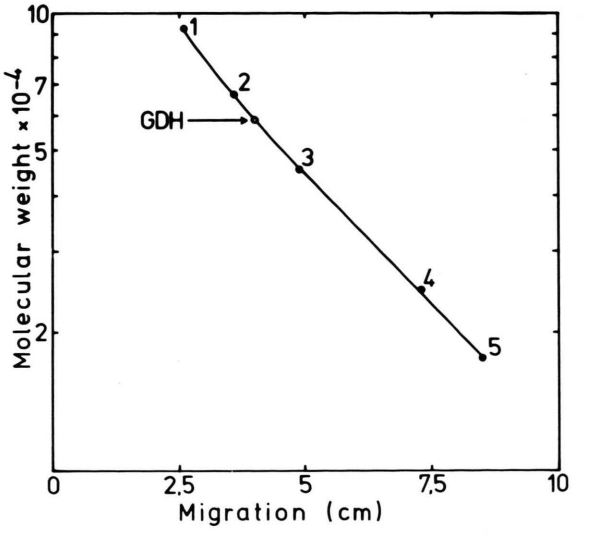

Fig. 4. Determination of the subunit molecular weight of glutamate dehydrogenase from *P. sativum* and *L. minor* by sodium dodecyl sulfate gel electrophoresis. Reference proteins: 1 = phosphorylase A (molecular weight 94000), 2 = bovine serum albumin (67000), 3 = ovalbumin (45000), 4 = chymotrypsinogen (25000), 5 = myoglobin (17800). GDH = position of the proteins of both plant enzymes.

mated for the subunit molecular masses of both enzymes (Fig. 4).

These results suggest that both glutamate dehydrogenases are tetramers with subunits of equal size. This could directly be proved by electron microscopic observation (Fig. 5). The micrographs of the negatively stained molecules show oligomers which are composed of four protomers arranged in a tetraedric structure with an edge-length of 95 Å.

Most glutamate dehydrogenases purified from non-plant sources are hexamers with subunit weights of 46000–55000 (see review [28]). However, re-

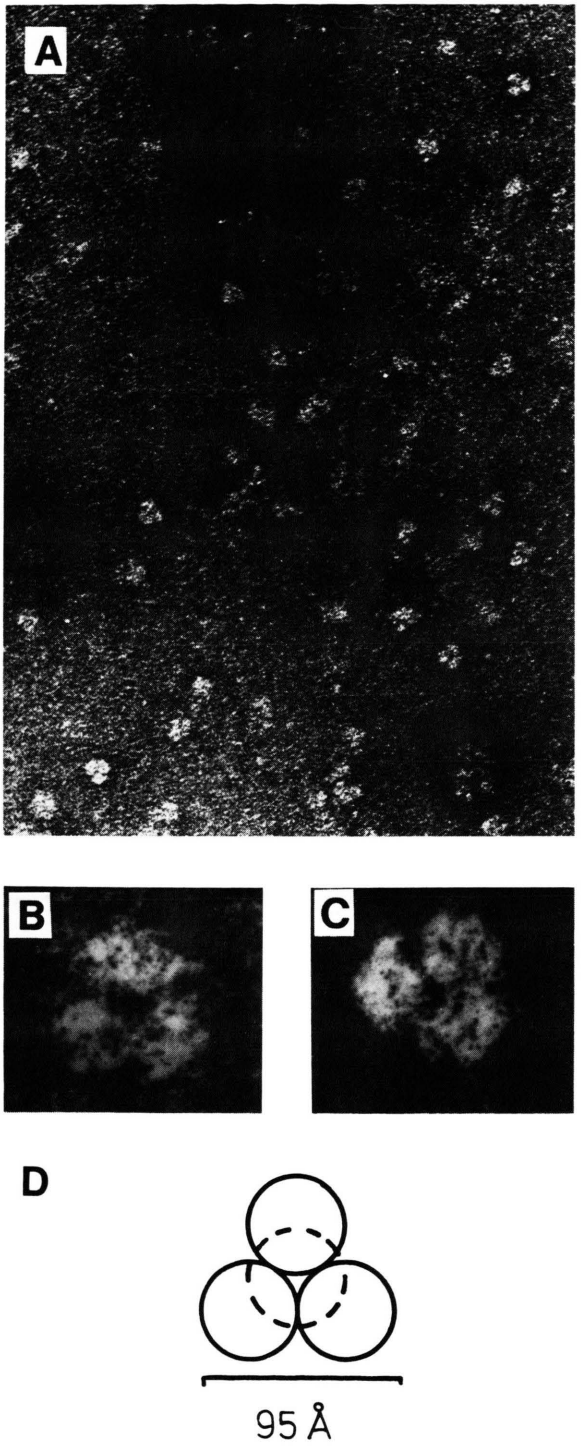

Fig. 5. Electron micrographs (A–C) of purified glutamate dehydrogenase from *L. minor*. D = Model of the glutamate dehydrogenase molecule showing a tetraedric structure composed of four subunits.

cently tetrameric enzymes have been isolated from *Neurospora* (subunit weight 116000) [29], *Chlorella* (45000) [30] and a *Halobacterium* (53500) [31]. Although the molecular weights of some plant glutamate dehydrogenases were determined ranging from 208000 to 250000 [1, 32, 33], the subunit composition has not been established. There is only one recent report suggesting the existence of a hexameric enzyme (molecular weight 270000) in the plant fraction of *Lupinus luteus* root nodules [5].

Amino acid composition

The amino acid composition of both plant enzymes calculated for a subunit weight of 58500 is shown in Table III together with the compositions of the hexameric enzymes from beef liver [34] and the tetrameric enzymes from *Chlorella* [30], *Neurospora* [29] and *Halobacterium* [31].

The amino acid compositions of the tetrameric plant enzymes show great similarities in comparison to the compositions of the beef liver enzyme (Table III) and various glutamate dehydrogenases from animals, fungi and bacteria [28]. In contrast the compositions of the tetrameric enzymes from *Halobacterium* and most expressed *Chlorella* are distinctly different. Of the tetrameric enzymes the *Neurospora*-enzyme having a subunit weight which is almost exactly twice as high as that of the plant glutamate dehydrogenase shows the greatest similarity with the plant enzymes in respect to the relative ratios of the individual amino acid residues. However, as established by sequence analysis the *Neurospora*-enzyme lacks extensive sequence homology with other glutamate dehydrogenases [35].

The N-terminal amino acids are alanine (*Pisum*-enzyme) and alanine or glycine (*Lemna*-enzyme).

Substrate specificity

The activities of the two purified enzymes with some alternative coenzymes and substrates are summarized in Table IV. Low but significant activities could be achieved especially for the *Lemna*-enzyme when NADH is replaced by NADPH, whereas substitution of NAD^+ by $NADP^+$ revealed less than 1% of the respective control activity for both enzymes. Similar results have been obtained using less purified preparations of the enzymes from *Lemna* [15] and *Pisum* [1]. Furthermore it should be mentioned that as with animal glutamate dehydrogenase the

Table III. Amino acid compositions of glutamate dehydrogenase from *L. minor* and *P. sativum* in comparison to the compositions of glutamate dehydrogenase from bovine liver [34], *Chlorella sorokiniana* (NAD-dependent enzyme) [30], *Halobacterium* (NADP-dependent enzyme) [31] and *Neurospora crassa* (NAD-dependent enzyme) [29].

Amino acid	Residues/subunit					
	L. minor [a] subunit wt. 58 500	*P. sativum* [a] subunit wt. 58 500	*Bovine liver* [b] subunit wt. 55 393	*Chlorella* [a] subunit wt. 45 000	*Halobacterium* [a] subunit wt. 53 000	*Neurospora* [a] subunit wt. 116 000
Asp/Asn	61 – 62	64	50	48	50	114
Thr	28	28	28	30	30	48
Ser	29	25	30	74	26	74
Glu/Gln	50	51	45	80	54	103
Pro	23	26	21	38	20	49
Gly	63 – 64	59	47	35	47 – 48	66
Ala	52	48	37	16	54	71
Val	35	37	34	8	40	69
Met	10	11	13	6	4	20
Ile	35 – 36	39	38	7	23 – 24	67
Leu	51	47	31	14	33 – 34	92
Tyr	9	12	18	7	22	41
Phe	21	19	23	6	15	47
His	11	12	14	8	3	25
Lys	36	40	33	27	13	65
Arg	30	24 – 25	30	6	19	63
Trp	n. d.	n. d.	3	6	7 – 8	7
Cys	n. d.	n. d.	6	6	3	9

[a] Tetrameric enzyme. [b] Hexameric enzyme. n. d. not determined.

Table IV. Substrate specificity of purified glutamate dehydrogenase from *L. minor* and seeds of *P. sativum*.

Substrate	Assay concentr. [mM]	Enzyme activity as % of control	
		L. minor	*P. sativum*
Complete assay		100	100
NADH omitted			
+ NADPH	0.21	7	1.5
NAD$^+$ omitted			
+ NADP$^+$	0.83	0.6	<0.1
2-Oxoglutarate omitted			
+ Glutarate	16.7	<0.1	<0.1
+ Oxaloacetate	16.7	0.1	<0.1
+ 2-Oxobutyrate	16.7	0.8	0.3
+ 2-Oxovalerate	16.7	3.2	1.6
+ 2-Oxoglutarate and 2-Oxovalerate	16.7 16.7	89.0	86.0
L-*Glutamate omitted*			
+ D-Glutamate	16.7	<0.1	0.1
+ D,L-Norleucine	33.4	1.0	1.5
+ D,L-Norvaline	33.4	5.6	2.0
+ D,L-Alanine	33.4	0.1	<0.1
+ L-Aspartate	16.7	<0.1	<0.1
+ D,L-2-Aminoadipate	16.7	<0.1	<0.1
+ L-Glutamate and D,L-Norvaline	16.7 16.7	65.0	98
Ammonium omitted			
+ L-Glutamine	83	1.0	0.2
+ Methylamine	83	<0.1	<0.1
+ Isoamylamine	83	<0.1	n. d.

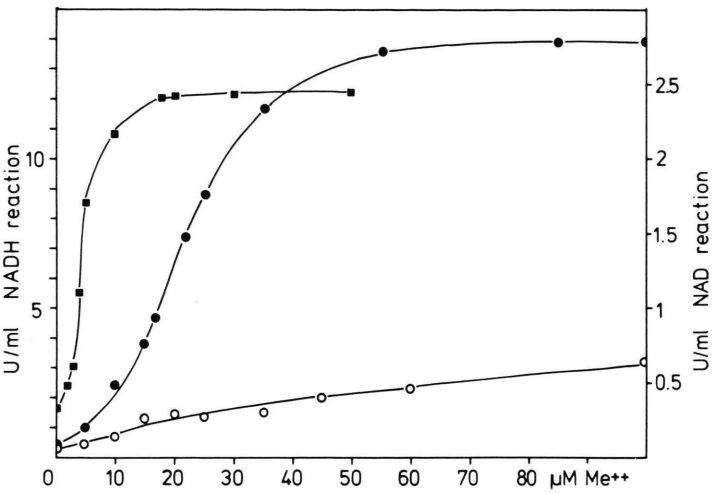

Fig. 6. Activation of purified EGTA-pretreated glutamate dehydrogenase from *L. minor* by divalent metal ions. ●—●: $+Ca^{2+}$ (NADH-dependent reaction); ■—■: $+Ca^{2+}$ (NAD^+-dependent reaction); ○—○: $+Zn^{2+}$ (NADH-dependent reaction).

pea-enzyme [36] and the *Lemna*-enzyme [37] are B-stereospecific in respect to the hydrogen transfer during NADH-oxidation. The small degree of activity with 2-oxobutyrate and 2-oxovalerate (substituted for 2-oxoglutarate) or norleucine and norvaline (instead of glutamate) is comparable to results reported for other enzymes. Neither glutamine nor monoamines could replace NH_4^+ as a substrate. Thus the substrate specificity of the plant enzyme seems to be similar to those reported for the enzymes from other sources [38].

Metal ion activation

The activation of the glutamate dehydrogenase catalyzed reactions by divalent metal ions has been studied previously using partially purified preparations of *L. minor* [15–16] and *P. sativum* [2, 14]. Some of these effects were reinvestigated using the purified enzymes.

The NADH-dependent reaction of the *Lemna*-enzyme purified in the absence of Ca^{2+} is reduced to about 50% whereas the NAD^+-dependent reaction is only slightly affected (0–20%). In the presence of 0.1 mM EDTA or EGTA the reactions are inactivated up to 90–98% (NADH-dependent) and 80–85% (NAD^+-dependent). Complete reactivation can be obtained upon addition of Ca^{2+} or to some extent Zn^{2+} (Fig. 6). The respective $K_{0.5}$ values for the Ca^{2+} activation are 22 µM (NADH-dependent reaction) and 4 µM (NAD^+-dependent reaction). Mg^{2+} is completely ineffective for both reactions when added instead of Ca^{2+}. This is in contrast to earlier studies where the oxidative desamination was found to be activated by either Ca^{2+} or Mg^{2+} [15, 16]. As in these studies partially purified enzyme preparations were used we assume that the activatory effect of Mg^{2+} was brought about by replacement of unspecifically protein-bound Ca^{2+} which in turn caused the activation.

The inactivation of the purified pea-enzyme by 0.1–1 mM EDTA is less complete: up to 50% (NADH-dependent reaction) and 20–30% (NAD^+-dependent reaction). Similar results are known from studies with less purified pea-enzyme [2]. Both reactions are completely reactivated by Ca^{2+}. For the NADH-dependent reaction a $K_{0.5}$ (Ca^{2+}) of 9.5 µM could be calculated.

Both purified enzymes show identical electrophoretical behaviour in the presence and absence of 0.1 mM EDTA or Ca^{2+}. Thus Ca^{2+} does not affect the enzyme aggregation as has been shown for glutamate dehydrogenase from *Blastocladiella* [39] and suggested for the pea enzyme [1], but instead Ca^{2+} seems to govern a reversible equilibrium between catalytically active and inactive enzyme forms as assumed from kinetic studies [16].

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