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GLUTAMATE DEHYDROGENASE OF *SPHAGNUM*

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Key Word Index—*Sphagnum fallax*; Sphagnaceae; Bryophyta; chloroplasts; mitochondria; L-glutamate dehydrogenase; [^{15}N]labelling; purification.

Abstract—Glutamate dehydrogenase (GDH) of *S. fallax* was purified 2000-fold to apparent homogeneity. As estimated by gel filtration and SDS-PAGE, a native molecular weight of M_r 210 000 and a subunit molecular weight of M_r 52 000 were determined, indicating that the enzyme is tetrameric with subunits of identical molecular weights. Isoforms could not be detected. Purified GDH displayed pH-optima of 8.6 with NAD(P)H and 7.7 with NAD^+ and a high K_m for ammonium of 28 and 41 mM with NADH and NADPH as coenzymes, respectively. The enzyme is located in the mitochondria. The occurrence of GDH activity in the cytosolic fraction was addressed to organelle breakage. Enhanced ammonium concentrations and a reduced carbon supply caused a substantial increase in GDH activity. Labelling studies with [^{15}N]ammonium and [^{15}N]glutamate were consistent with the role of the enzyme in the oxidative deamination of glutamate. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Glutamate dehydrogenase, GDH (EC 1.4.1.2-4), has been found in almost all organisms examined. The enzyme catalyses the reversible reductive amination of 2-oxoglutarate to L-glutamate, which has long been considered as a major route of ammonium assimilation in plants. It is generally accepted that the glutamine synthetase/glutamate synthase cycle represents the major pathway for the assimilation of ammonium by higher plants [1]. However, it is still discussed that GDH acts as an alternative to the glutamine synthetase/glutamate synthase cycle under conditions of high ammonium concentrations, stress, or senescence [2–4]. Recent work employing *in vivo* and *in vitro* methods demonstrated that GDH is involved in the oxidative deamination of glutamate to provide carbon skeletons to the citric acid cycle [5–7].

GDH has been purified and characterized in animals, microorganisms and higher plants, but until now little information is available concerning this enzyme in bryophytes. Screening of more than 20 bryophytes revealed GDH activity in crude extracts of all species, but failed to detect the enzyme in *Sphagnum* initially, suggesting that GDH may be lacking in this genus [8]. In the meantime NADP(H)- and NAD(H)-dependent GDH activity has been detected in crude extracts of several *Sphagnum* species with a 10-fold enhanced enzyme activity in mosses cultivated axenically in continuous feed bioreactors [9]. The addition of high concentrations of ammonia (40 mM) to the culture medium caused an increase in specific GDH activity [10]. But the assay of GDH in crude extracts is strongly affected by side reactions [11]. Thus, a more detailed characterization was necessary to evaluate the properties of the enzyme in *Sphagnum*.

In this paper we describe the purification, characterization, and compartmentalization of GDH from *S. fallax*. Furthermore, we report on the effects of ammonium- and CO_2-supply on extractable GDH activity. Studies with [^{15}N]ammonium and [^{15}N]glutamate were carried out to evaluate the metabolic function of GDH *in vivo*.

Abbreviations: AOA, aminooxyacetic acid; BSA, bovine serum albumin; CCO, cytochrome-c oxidase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GDH, glutamate dehydrogenase; HEPES, *N*-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid; IRMS, isotope ratio mass spectrometry; LDH, lactate dehydrogenase; MOPS, 3-(*N*-morpholino)propanesulphonic acid; MSO, methionine sulphoximine; PMSF, phenylmethylsulphonyl fluroide; PVP, polyvinylpyrrolidone.

RESULTS

Purification of GDH

Side reactions, which affected the GDH assay in crude extracts [11], could be completely eliminated

Table 1. Purification of GDH from *S. fallax*. Values are representative of four separate experiments

Purification step	Total activity (nkat)	Total protein (mg)	Specific activity (nkat mg^{-1})	Purification (-fold)	Yield (%)
Crude extract	123	365	0.34	1	100
30% ammonium sulphate	85.0	292	0.31	0.9	69
Phenyl Sepharose	46.1	3.6	12.5	37	37
Superdex 200 (first)	30.6	0.25	126	371	25
Q Sepharose	10.8	0.05	240	706	8.8
Superdex 200 (second)	9.2	0.02	681	2003	7.5

by gel filtration. For this reason crude extracts were generally desalted on a Sephadex G 25 column.

The purification protocol developed for GDH of *S. fallax* is summarized in Table 1. The catalytic activity of the enzyme was determined with NADH as coenzyme. By far the most effective purification step was the elution from the Phenyl Sepharose column by a two step gradient, which removed about 99% of the contaminating proteins and resulted in a 37-fold increase in specific GDH activity. Active fractions of four runs were collected and concentrated. After running the Superdex 200 column, a 371-fold purification was obtained with a yield of 68% of the loaded activity. This high recovery was achieved by the addition of 4 mM NH_4Cl, 2 mM $CaCl_2$, and 1 mM 2-oxoglutarate to the buffer systems. Without these components more than 90% of the applied enzyme activity was lost during elution.

The five step purification protocol yielded about 20 μg of protein with a final recovery of 7.5% of the initial GDH activity of the crude extract. The purified enzyme displayed a specific activity of 681 nkat mg^{-1} protein. After this final purification step, one single polypeptide was detected on silver-stained SDS-PAGE (Fig. 1), indicating that the enzyme has been purified to apparent homogeneity.

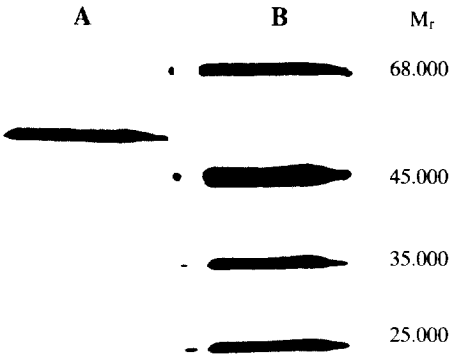

Fig. 1. SDS-PAGE of purified GDH from *S. fallax* (lane A). The molecular weights of the marker proteins (lane B) are given in the margin. Proteins were visualized by siler staining.

Characterization of purified GDH

It was observed during enzyme purification that the reductive amination of 2-oxoglutarate could be catalysed by the enzyme preparation in the presence of either NADH or NADPH. The ratio of NADH–NADPH-dependent activities was 1:1.6, and remained reasonably constant during all purification steps. These data suggest a dual coenzyme specificity of the *Sphagnum* GDH. The oxidative deamination of glutamate could be determined by the enzyme preparation in the presence of NAD^+ only. With $NADP^+$ as coenzyme the GDH activities were too low for accurate assay, even if concentrated enzyme preparations were used. *In vitro*, at optimum assay conditions, the ratio of the reductive amination to the oxidative deamination was found to be 2.4 to 1 for the NAD(H)-dependent GDH activity.

During each purification step GDH activity eluted as one single peak. Similarly, native PAGE showed only one protein band coincident with GDH activity. The native molecular weight of the purified GDH was determined by gel filtration on a Superdex 200 column. The eluted activity peak corresponded to a native molecular weight of 210 000 as estimated by comparison to standard molecular weight markers. By SDS-PAGE, a subunit molecular weight of 52 000 was determined (Fig. 1), indicating that the enzyme is tetrameric with subunits of identical molecular weights.

The pH dependence of the enzyme was examined over the range of pH 6.8–7.8 in 100 mM imidazole–HCl and over the range of pH 7.7–9.1 in 100 mM Tris–HCl. The pH optima for the aminating and deaminating reactions of the purified GDH were observed to be 7.7 with NAD(P)H and 8.6 with NAD^+ as coenzymes, respectively.

The K_m values for different substrates of GDH were determined from the Lineweaver–Burk plots at the pH optimum for the reaction (Table 2). All substrate saturation curves followed normal Michaelis–Menten kinetics. Concentrations of glutamate above 25 mM resulted in a considerable inhibition of enzyme activity. The *Sphagnum* GDH, comparable to many other plants, displayed a high K_m for ammonium of

Table 2. Michaelis constants for different substrates of GDH isolated from *S. fallax*

Reaction catalysed	Substrate	K_m (M)
NADH-dependent	NADH	8.3×10^{-6}
	2-Oxoglutarate	0.7×10^{-3}
	NH_4Cl	2.8×10^{-2}
NADPH-dependent	NADPH	1.1×10^{-4}
	2-Oxoglutarate	6.3×10^{-3}
	NH_4Cl	4.1×10^{-2}
NAD^+-dependent	Glutamate	2.1×10^{-3}
	NAD^+	1.4×10^{-4}
$NADP^+$-dependent	Glutamate	ND
	$NADP^+$	ND

ND—not detectable.

28 mM and 41 mM with NADH and NADPH as coenzymes, respectively (Table 2). The K_m value for NADH was 13 times lower than that for NADPH.

Compartmentalization of Sphagnum *GDH*

We studies the subcellular localization of GDH in purified chloroplasts, purified mitochondria, and a cytosolic fraction (Table 3). The purity of the organelles was assessed by the activities of the marker enzymes cytochrome-c oxidase (CCO), lactate dehydrogenase (LDH), and chlorophyll as a marker of plastids. Chloroplasts and mitochondria seemed to be free of cytoplasmic contamination, as LDH activity was not detectable. The low level of total CCO activity of 1.2% in the chloroplastic fraction indicated a minor mitochondrial contamination. Similarly, the mitochondrial fraction only contained negligible amounts (0.8%) of the total chlorophyll.

Compartmentalization experiments with both NADH and NADPH as coenzymes revealed that GDH activity could not be related to purified chloroplasts. 90.8% of the total GDH activity was found in the cytosolic fraction. Compared to the cytosol, a 13-fold increase in specific activity was obtained in purified mitochondria. The CCO activity displayed a similar subcellular distribution as GDH, with 87.6% of the total activity located in the cytosolic fraction and a 16-fold increase in specific activity in purified mitochondria. The consistent correlation between GDH activity and CCO activity suggests a mitochondrial localization of the enzyme from *S. fallax*, though the main part of GDH was assayed in the cytosolic fraction, but this activity seems to be due to organelle breakage.

Effect of a reduced CO_2*-supply on GDH activity*

The submersed culture of *Sphagnum* species in continuous feed bioreactors makes it feasible to vary the CO_2 concentration supplied to the mosses, under well-defined conditions. If the CO_2 concentration of the air supplied to the bioreactors was reduced from 0.5% (v/v) to 0.03% (v/v) for 7 days, a cessation of growth takes place. GDH levels increased during the first three days and remained at this higher level (Fig. 2). Specific activities of NADH-GDH and NADPH-GDH increased by a factor of 2.7 (0.6 nkat mg^{-1}) and 3.5 (1.3 nkat mg^{-1}), respectively. From day 7 to day 14, at restored CO_2 concentrations of 0.5% (v/v), the

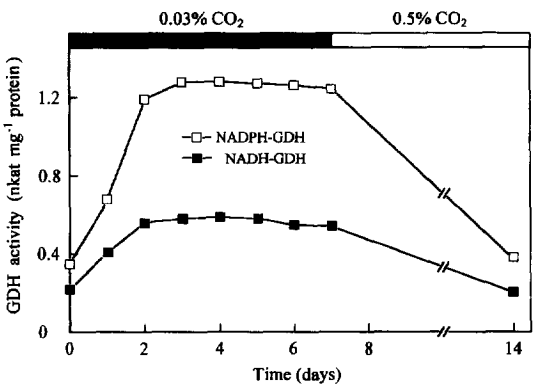

Fig. 2. Effect of varying CO_2-concentrations on GDH activity over a 14 day period. At day 7 the CO_2-concentration was restored to 0.5% (v/v). The values are representative of three independent experiments.

Table 3. Subcellular distribution of GDH (NADH as coenzyme), marker enzymes for mitochondria (cytochrome-c oxidase, CCO), cytosol (lactate dehydrogenase, LDH), and chlorophyll as a marker for plastids from *S. fallax*. Experiments with NADPH as coenzyme revealed similar results. Enzyme activities are expressed as percentage of recovered total activity or as volume activity

Fraction	Activity (nkat ml^{-1}) GDH	LDH	CCO	Chlorophyll ($\mu g\ ml^{-1}$)
Cytosol	0.3±0.05	0.3±0.1	1.6±0.4	6.4±0.8
Purified mitochondria	4.2±0.42	ND	25.6±2.8	8.3±0.6
Purified chloroplasts	ND	ND	2.8±0.3	181 ±17.3
	Per cent total activity			*Per cent total chlorophyll*
Cytosol	90.8	100	87.6	79.7
Purified mitochondria	9.2	ND	11.2	0.8
Purified chloroplasts	ND	ND	1.2	19.5

ND—not detectable.

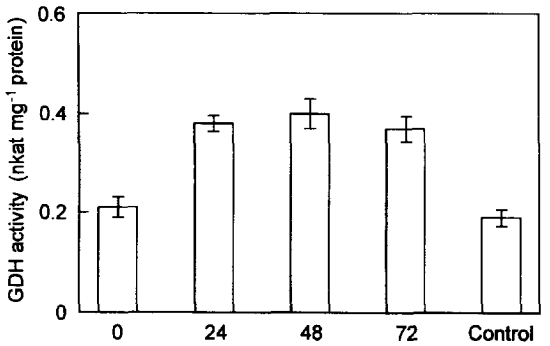

Fig. 3. Effect of an enhanced ammonium supply (1.9 mM) on GDH activity in crude extracts of *S. fallax* during a 72 hr period. The values represent the mean ± SE of three independent experiments.

GDH level decreased continuously to a value near the onset of the experiment. Specific glutamine synthetase activity was affected in the opposite way, dropping to a value of 50% of the initial activity within 24 hr. The activity remained at this low level until the supplied air was enriched with 0.5% (v/v) CO_2 again (Kahl, pers. comm.).

Effect of ammonium on GDH activity

Reduced ammonium concentrations (0.1 mM) during a 72 hr treatment had almost no effect on GDH activity. As opposed to this an enhancement of the ammonium concentration in the nutrient solution from 0.38 to 1.9 mM caused an increase in GDH activity from 0.2 mg^{-1} protein to 0.4 nkat ml^{-1} within 24 hr (Fig. 3), which remained constant at this higher level. The control plants, provided with 0.38 mM ammonium, showed no increase in GDH activity during the experiment.

[^{15}N]-*labelling studies*

The fate of [^{15}N]-labelled ammonium in the presence and absence of the glutamine synthetase inhibitor MSO was followed by *in vivo* NMR during a 12 hr period. [^{15}N]Ammonium was rapidly incorporated into the glutamine amide group and later appeared as amino nitrogen of glutamate. MSO-treated samples showed no incorporation of [^{15}N] label into glutamine, glutamate, or any other amino acid (data not shown), indicating that GDH is not involved in ammonium assimilation in *S. fallax* under the conditions used. To investigate a deaminating function of *Sphagnum* GDH, the catabolism of [^{15}N]glutamate in mosses pretreated with 1.5 mM MSO and 1.5 mM AOA was investigated. After an 18 hr incubation period in 10 mM [^{15}N]glutamate the ammonium content increased to 18.5 μg ml^{-1}, as compared to 6.1 μg ml^{-1} in the control. Analysis of the [^{15}N]enrichment revealed, that 54% (6.7 μg ml^{-1}) of that increase can be accounted for by the breakdown of glutamate. In the control treatment no [^{15}N]ammonium could be detected. These data provide strong evidence for a catabolic role of GDH in *Sphagnum*.

DISCUSSION

The purification procedure achieved for *S. fallax* resulted in a 2000-fold purification to homogeneity with a specific activity of 681 nkat mg^{-1} protein and yielded about 200 μg of the purified protein. As estimated by gel filtration and SDS-PAGE, the purified GDH has a native M_r of 210 000 with subunits of M_r 52 000, indicating that the enzyme is composed of four subunits of identical molecular weight. The obtained M_r resemble those of plant GDHs, which generally display native molecular weights in the range 208 000–270 000 with subunits in the range of M_r 46 000–58 500 and are composed of four or six subunits [3].

Usually plant GDHs contain a number of isoenzymes, at most 17 in shoots of pea [12]. The isozymic number varies not only with plant species, but also within one species, caused by nutritional and environmental conditions [3]. The algae *Chlorella* displayed only one constitutive GDH, whereas in this species two other GDHs were synthesized *de novo* in the presence of ammonium ions [13, 14]. In *S. fallax*, no GDH isoenzymes could be detected under the culture conditions described.

GDH isolated from *S. fallax* can use NADH as well as NADPH as coenzymes, but an oxidative deamination of glutamate was only measurable in the presence of NAD^+. The same phenomenon has been described for *Vicia faba* [15] and GDH-2 of *Glycine* [16]. In contrast to most plants, in *S. fallax* NADPH-dependent GDH activity was higher than that with NADH as coenzyme. These data confirm our previous results [9], and results obtained for *S. imbricatum* [10]. In the latter species the NADPH-GDH activity was 5–10 times higher than that with NADH as coenzyme. On the other hand, in *S. fallax* the K_m value for NADH was 13 times lower than that for NADPH, indicating a higher affinity of the enzyme for NADH.

Of importance to the metabolic role of GDH is the apparent high K_m of the enzyme for the substrate ammonium, which in *Sphagnum* amounts to 28 and 41 mM for NADH-dependent GDH and NADPH-dependent GDH, respectively. The high K_m for ammonium has often been used to question an ancillary role of the enzyme to the glutamine synthetase/glutamate synthase pathway in the assimilation or reassimilation of ammonia in plant tissues [3].

In contrast to many higher plants [17–20] no GDH activity could be related to purified chloroplasts of *S. fallax*. Significant activity was found in the cytosolic as well as in the mitochondrial fraction. We assign the high amount of approximately 90% of the total GDH activity assayed in the cytosolic fraction to unavoidable organelle breakage, which occurs due to the comparatively rigorous technique which is necessary to distrupt the robust cell walls of the *Sphagnum* plant-

lets. The occurrence of about 80% of the total CCO activity in the cytosolic fraction indicates that the majority of the mitochondria were broken during homogenization. However, in purified mitochondria of *S. fallax* a strong enrichment (13-fold) of GDH activity was obtained, correlating with the increase of specific CCO activity in this organelle fraction. The consistent correlation between GDH activity and the mitochondrial marker enzyme clearly points to a mitochondrial localization of the *sphagnum* GDH.

The occurrence of GDH activity in the cytosolic fraction in addition to mitochondria has been reported for several plants [21]. If these enzymes, obtained from both fractions, displayed similar physical and kinetic properties, it is also suggested that the cytoplasmic activity is due to broken mitochondria.

In *S. fallax*, comparable to most plants investigated, the presence of enhanced ammonium concentrations cause an increase of GDH activity. Only in a few plant species has ammonium either no effect or even inhibits the enzyme activity [3]. This increase in GDH activity could be attributed either to ammonium indiction of GDH as reported for *Avena* [22] and *Triticum* [23] or by modulating the activity of existing enzyme molecules.

Depletion of the carbon source leads to a cessation of growth and simultaneously induces a substantial increase in GDH activity in *S. fallax*. Under this condition protein synthesis is restricted due to a lack of carbon skeletons. Protein catabolism releases glutamate, which could be oxidized by GDH to 2-oxoglutarate with the concurrent release of ammonium, which could be reassimilated by glutamine synthetase. The mitochondrial location and the increase in activity under carbon limiting conditions suggests that the main function of the enzyme in *Sphagnum* comparable to the enzyme in suspension cultures of carrot [5, 6] and corn seedlings [7], is deaminating glutamate to provide carbon skeletons for the citric acid cycle under conditions of carbon limitation.

We used a combination of *in vivo* and *in vitro* methods to investigate the function of GDH in *Sphagnum*, and the results were entirely consistent with a role for the enzyme in the oxidative deamination of glutamate. Analysis of the assimilation of [^{15}N]ammonium using *in vivo* [^{15}N] NMR spectroscopy in the presence and absence of the GS inhibitor methionine sulphoxime showed that GDH played no part in the assimilation of ammonium which occurred via the glutamine synthetase/glutamate synthase cycle. Additionally, the catabolism of [^{15}N]glutamate was studied and a substantial proportion of the label was released as [^{15}N]ammonium under conditions that prevented its reassimilation.

EXPERIMENTAL

Plant material. Sphagnum fallax (Klinggr.) Klinggr. clone 1 was initiated from a spore according to ref. [23]. The mosses were cultivated under axenic conditions in 6 l continuous feed bioreactors aerated with air containing 0.5% (v/v) CO_2 [24]. A four-fold concd standard nutrient soln was applied at a flow rate of 100 ml hr^{-1} [25].

Enzyme assays. The catalytic activity of glutamate dehydrogenase in the aminating direction was monitored by the decrease in A_{340}. The assay system contained 10 mM 2-oxoglutarate, 0.2 mM NAD(P)H, 150 mM NH_4Cl, 100 mM Tris–HCl (pH 7.7), and 200 μl of enzyme solution in final volume of 1.05 ml. The oxidative deamination of glutamate was assayed at pH 8.6 by measuring the increase in A_{340} associated with NAD(P) reduction. The assay system contained 50 mM L-glutamate, 2 mM NAD(P)$^+$, 100 mM Tris–HCl (pH 8.6), and 200 μl of enzyme soln in a final vol. of 1.05 ml.

Cytochrome-c oxidase activity (EC 1.9.3.1) was assayed spectrophotometrically at 550 nm according to the procedure of ref. [26], and lactate dehydrogenase (EC 1.1.1.27) was monitored at 340 nm by the oxidation of NADH in the presence of pyruvate [27]. All enzyme activities are given as nanokatals (nkat).

Preparation of crude extracts. The enzyme prepn procedures and all subsequent purification steps were performed at 4° unless otherwise indicated. Most of the water adhering to the mosses was removed by centrifugation at 200 *g* for 5 min. Approximately 5 g of plant material was homogenized in 25 ml of 50 mM Tris–HCl (pH 8.5), 5 mM DTT, 1 mM PMSF (buffer A) in a cell homogenizer with CO_2 cooling (MSK Braun, Melsungen, Germany). The homogenate was squeezed through a nylon mesh (80 μm pore size) and centrifuged at 30 000 *g* for 20 min. The supernatant was desalted on a Sephadex G25 column (1.6 × 5 cm, Pharmacia, Freiburg, Germany) and used directly for enzyme assays.

Glutamate dehydrogenase activities at different CO_2 and ammonium concentrations. To examine the influence of a reduced carbon supply to the mosses, the bioreactors were supplied with air in which the part of the CO_2 was reduced from 0.5% (v/v), to 0.03% (v/v).

To examine the response of GDH to different ammonium concns the precultivated mosses were harvested from the bioreactors, sepd from the external medium, and equal amounts of 4 g fr. wt (0.5 g dry wt) applied to four bioreactors with a capacity of 480 ml. To induce a reduced ammonium availability, three bioreactors were provided with a modified four-fold standard nutrient soln containing 0.1 mM ammonium. The fourth bioreactor was provided with a four-fold standard nutrient soln (0.38 mM ammonium) and served as control. The nutrient solns were applied at a flow rate of 10 ml hr^{-1}. The first bioreactor was harvested after 24 hr, the second after 48 hr, and the third as well as the control after 72 hr and the mosses assayed for GDH activity. An enhanced ammonium availability was established by providing the mosses with a modified standard nutri-

ent solution containing 1.9 mM ammonium, according to the procedure described above.

[^{15}N]-*labelling studies. In vivo* ^{15}N NMR studies were performed according to ref. [5]. The initial extracellular concentration was 20 mM for the labelled ammonium chloride. To investigate the catabolism of [^{15}N]glutamate equal amounts of 3 g fresh weight of the precultivated plant material were applied to two bioreactors with a capacity of 480 ml. One bioreactor containing 3 g of inactivated (100°, 3 min) plant material served as control. Each bioreactor was provided with 200 ml of a modified four-fold standard nutrient solution containing 10 mM [^{15}N]glutamate (98% [^{15}N] enrichment) as the sole nitrogen source, 1.5 mM MSO, and 1.5 mM AOA. After an 18 hr incubation period the mosses were harvested, denatured (100°, 3 min), homogenized in 15 ml aq. bidest containing 1 M KCl to displace ammonium from the cation exchange sites, and centrifuged at 30 000 *g* for 20 min.

The ammonium content of the plant extracts was analysed employing cellophane dialysis and the indophenol blue reaction for ammonium detection (Traacs, Bran and Lübbe). An improved microdiffusion-technique was used to determine the ^{15}N-enrichment of ammonium (Goerges and Dittert, unpublished). Briefly, extracts were transferred to 20 ml polypropylene vials, saturated with MgO, and ammonia was dialysed through PTFE membranes into an acid trap (N-free filter paper impregnated with 10 μl of 2.5 mM $KHSO_4$) for 2 days. The filter paper discs were subsequently dried in an ammonia-free atmosphere. The determination of the ^{15}N-enrichment was carried out using a continuous flow elemental analyser (Carlo-Erba) linked to an isotope ratio mass spectrometer (Delta C, Finnigan MAT). Considering the total ammonium content of the extract and the ^{15}N-enrichment of the glutamate used (98%), the extent of glutamate breakdown was calculated.

Purification of glutamate dehydrogenase. The plant material (about 90 g) was homogenized in 450 ml of buffer A in a Waring blender at high speed for 1 min, passed through four layers of miracloth and centrifuged at 30 000 *g* for 20 min. The supernatant was brought to 30% saturation with ammonium sulfate. The mixt. was stirred for 30 min and then centrifuged at 30 000 *g* for 20 min to remove precipitated proteins. Subsequently the supernatant was applied to Phenyl Sepharose columns (1.6 × 14 cm, Pharmacia) previously equilibrated with buffer A containing 500 mM ammonium sulphate. Bound proteins were eluted with the discontinuous gradient of 120 and 10 mM ammonium sulphate in buffer A at a flow rate of 50 ml hr^{-1}. The buffers were changed after the absorbance returned to baseline. Frs of 10 ml were collected and those containing glutamate dehydrogenase activity were pooled and concentrated in a Centriprep Concentrator (Amicon, Witten, Germany) to a final volume of 3 ml. This concentrate was loaded onto a Superdex 200 column (1.6 × 60 cm, Pharmacia) previously equilibrated with buffer B (4 mM NH_4Cl, 2 mM $CaCl_2$, 1 mM 2-oxoglutarate, 1 mM PMSF, and 50 mM Tris; the pH was adjusted to 8.5 with HCl). Bound proteins were eluted with buffer B at a flow rate of 12 ml hr^{-1} at 10°. Frs (3 ml) containing glutamate dehydrogenase activity were pooled and applied to a Q Sepharose column (1 × 4 cm, Pharmacia), equilibrated with buffer C (50 mM Tris–HCl, pH 8.5). Proteins were eluted with a linear gradient of NaCl from 0 to 400 mM dissolved in buffer C. Active frs (3 ml) were pooled, concd to a final vol. of 2 ml and loaded onto a Superdex 200 column previously equilibrated with buffer B for a second time. The elution was performed with buffer B as described above. Frs containing glutamate dehydrogenase activity were pooled and used for further analysis.

Electrophoresis. SDS-PAGE was carried out using 10% polyacrylamide (Serva) slab gels adjusted to pH 8.8 with stacking gels adjusted to pH 6.8 and buffer systems according to the method of [28]. Proteins were visualized by silver staining [29].

Protein determination. Protein concentrations were established using the Bio-Rad protein dye reagent [30] with bovine serum albumin as standard.

M_r *determination.* The M_r of the native protein was determined by gel filtration on a Superdex 200 (1.6 × 60 cm, Pharmacia) at a flow rate of 12 ml hr^{1}. The standards used for calibration were ferritin (M_r 450 000), catalase (M_r 240 000), aldolase (M_r 158 000), BSA (M_r 68 000), ovalbumin (M_r 45 000), chymotrypsinogen (M_r 25 000), and cytochrome-c (M_r 12 500). The void volume (V_0) was determined with Blue Dextran (M_r of 2×10^6).

The M_r of the protein subunits was determined by comparison to standards run on the SDS-PAGE system. The markers included: BSA (M_r 68 000), ovalbumin (M_r 45 000), lactate dehydrogenase (M_r 35 000), and chymotrypsinogen A (M_r 25 000).

Subcellular fractionation experiments. Chloroplasts and mitochondria were isolated as described previously [31, 32].

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