Glutamate Dehydrogenase from *Pisum sativum* L.*

Localization of the Multiple Forms and of Glutamate Formation in Isolated Mitochondria

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Abstract. A 2-8-fold increase in the activity of glutamate dehydrogenase (GDH), accompanied by an alteration of the GDH isoenzyme pattern, was observed in detached pea shoots floated on tap water (preincubated shoots). Sugars suppressed the process, whereas NH₄⁺ and various metabolites as well as inhibitors of energy metabolism and protein synthesis were ineffective. The subcellular distribution pattern revealed evidence that the GDH isoenzymes are exclusively located in the mitochondrial matrix. The alterations in GDH activity occurring in preincubated shoots are restricted to the mitochondria.

An experimental device suitable for studying the GDH function in isolated intact mitochondria has been established. Using [14C] citrate as the carbon source and hydrogen donor, the mitochondria synthesized considerable amounts of glutamate upon addition of NH₄⁺. The rates of glutamate formation in dependency of increasing NH₄⁺ levels follow simple Michaelis-Menten kinetics. Half-saturation concentrations of NH₄⁺ of 3.6 ± 1.2 mM; 1.9 ± 0.06 mM and 1.6 ± 0.1 mM were calculated for the mitochondria isolated from pea shoots, roots, and preincubated shoots, respectively. The results are discussed in relation to the possible role of GDH in NH₄⁺ assimilation at elevated intracellular NH₄⁺ levels.

Key words: Glutamate dehydrogenase – Glutamate formation – Mitochondria – Isoenzyme patterns – *Pisum*.

* Dedicated to Professor Dr. Maximilian Steiner on the occasion of his 75th birthday

Introduction

NAD-dependent glutamate dehydrogenase (GDH; EC 1.4.1.2.) is known to form complex isoenzyme patterns in a great number of plants (Thurman et al. 1965; Yue 1969; Errel et al. 1973; Lee 1973; Ratajczak et al. 1977). The isoenzymes of *Pisum sativum* constitute tissue-specific patterns (Pahlich 1972; Hartmann 1973). One pattern (GDH-I) is found in the cotyledons and young shoots. The other one (GDH-II) occurs, in addition to the GDH-I isoenzymes, in roots and older shoots. In young shoots the formation of GDH-II isoenzymes can be induced by removal of the cotyledons.

The mitochondrial localization of plant NAD-dependent GDH has been demonstrated by a number of authors; evidence, however, for distinct cytosolic isoenzymes has also been presented (s. Miflin and Lea 1977).

Recent research has shown that, as in bacteria, the assimilation of inorganic nitrogen in plants occurs mainly via the glutamine synthetase/glutamate synthase pathway ("GS/GOGAT-pathway") (s. Miflin and Lea 1976; Hartmann 1976), leaving the physiological function of GDH an open question.

NAD-dependent GDH is the only enzyme involved in the interconversion of ammonium and organic nitrogen known to occur in the mitochondria. It was our intention to establish an experimental device suitable for studying the metabolic participation of GDH in isolated intact mitochondria.

*Pisum sativum* was selected because the pea GDH is one of the most frequently studied enzymes and various in vitro data are available. Here we describe the preparation of mitochondria from pea shoots with GDH-I as well as induced GDH-II isoenzyme patterns, their subcellular distribution, and the kinetics of glutamate formation in isolated mitochondria.

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Materials and Methods

Cultivation and Preparation of Plant Materials

Pea seeds (*Pisum sativum* L. "Überreichen") were purchased from Samenhaus Schnitzius (Bonn, F.R.G.). Seedlings were grown in moist sand in the greenhouse. Etiolated seedlings were grown in the dark at 20–22°C. Six to eight day-old seedlings were harvested and the shoots were detached approx. 5 mm above the insertion of the cotyledons. The detached shoots were either immediately used for particle isolation and enzyme extraction (designated as "control shoots") or floated on tap water for 18 h and then used as described above (referred to as "preincubated shoots"). In some experiments roots of 6–8 day-old seedlings were used for comparison.

Preparation of GDH Extract

The acetone powder of plant organs were prepared as described in Ehmke and Hartmann (1976). The acetone powder (50–100 mg) was extracted with 10 ml 0.1 M Tris-HCl buffer, pH 8.2, for 20 min at 4°C and centrifuged at 30,000 g. The supernatant was dialyzed against running tap water for 20 h and used for enzyme assays.

Enzyme Assays

All assays were performed at 25°C. When subcellular particle fractions were used, 0.02 ml of 20% Triton X-100 was added to the assay. All enzyme activities are expressed in units (U); 1 U catalyzes the conversion of 1 nmol substrate per min.

GDH activity was assayed according to Ehmke and Hartmann (1976). The standard assay system (total volume 1.2 ml) contained the following components: NADH-dependent reaction: 0.1 ml enzyme extract; 0.8 ml 0.1 M Tris-HCl buffer, pH 7.8, containing 1.5 mM CaCl₂; 1.0 ml 25 mM NADH; 1.0 ml 0.2 M oxoglutarate. NAD⁺-dependent reaction: 0.1 ml enzyme extract; 0.9 ml 0.1 M Tris-HCl buffer, pH 8.5; 0.1 ml 25 mM NAD⁺; 0.1 ml 0.4 mM L-glutamate. NADH and NAD⁺ were adjusted to pH 7.0 with Tris-HCl buffer. Appropriate blank controls were performed, omitting individual substrates.

MDH (EC 1.1.1.37) was assayed according to Hock (1973), GOT (EC 2.6.1.1) according to Bergmeyer and Bernt (1974), fumarase (EC 4.2.1.2) according to Hill and Bradshaw (1969) and SDH (EC 1.3.99.1) with slight modifications according to Kolloeffel (1970)

Preparation of Mitochondria

All steps were performed at 0–4°C. Composition of the "isolation medium": 0.5 M mannitol; 0.07 M sucrose; 0.02 M HEPES; 0.02 M KH₂PO₄; 0.3 mM EDTA; 0.3% BSA; 5 mM cysteine. BSA and cysteine were added immediately before use; the pH was adjusted to 7.3. "Wash medium": 0.4 M mannitol; 0.07 M sucrose; 0.02 M HEPES; 0.02 M KH₂PO₄; 0.1% BSA; 0.02 M HEPES; final pH 7.1.

Precooled "control shoots" or "preincubated shoots" or "roots" (100 g fresh weight each) were ground in a mortar with 200 ml of isolation medium. The mazerate was squeezed through a layer of cheese cloth and centrifuged at 1,250 g for 10 min using a RC-5 Sorvall centrifuge and a SS 34 rotor (DuPont, Bad Nauheim/F.R.G.). The pellet was discarded and the supernatant centrifuged at 12,500 g for 10 min. The resulting supernatant was designated as the "soluble fraction". The pellet "crude mitochondria" was resuspended in 5 ml of wash medium.

Density Gradient Centrifugation

An aliquot of the washed mitochondria (1.5 ml corresponding to 4–5 mg protein) was layered on a linear sucrose density gradient (38 ml, 30 to 60 w/v) containing 5 mM EDTA; 2.5 mM MgCl₂ and 10 mM HEPES, pH 7.1. Centrifugation was performed at 23,000 rev min⁻¹ for 270 min in a L-27SB type ultracentrifuge (Beckmann, München/F.R.G.), using a SW-27 rotor. For fractionation, 1.8 ml fractions were drained from the bottom of the gradient.

Respiratory Measurements

Mitochondrial oxygen consumption was measured at 25°C in a 1.4 ml stirred "Perpet" cell (Oxygraph 22, Oxygraphic). Mitochondrial respiration was measured in a Clark type oxygen electrode, E012 (WTB, Weilheim/F.R.G.). The reaction medium (identical with the "wash medium") contained 0.5–0.8 mg of mitochondrial protein.

Tracer Experiments

Standard assay conditions: 1.5 ml of reaction medium, containing 1 mM [1,5-14C] citrate (9.4·10⁵ Bq) and 2.3-3 mg of mitochondrial protein, and additions (i.e. NH₃) as indicated, were incubated aerobically at 25°C for 20 min. The incubation was terminated by addition of 5 ml of hot 70% ethanol, and the residue was removed by centrifugation.

The ethanolic extract was separated in an "amino acid fraction" and an "organic acid fraction" by ion exchange chromatography on Amberlite IR-120 (Serva, Heidelberg/F.R.G.), according to Eschrich and Hartmann (1969). The amino acids were separated by thin-layer chromatography on silica gel 60 (Merck, Darmstadt/F.R.G.) with the solvent 96% ethanol/25% NH₃ (70/35); detection: ninhydrine reagent (Stahl 1967). The Krebs cycle acids were separated on cellulose plates (Merck, Darmstadt/F.R.G.) with the solvent 96% ethanol/25% NH₃ (70/35); detection: ninhydrine reagent (Stahl 1967). Detection: brom cresol green reagent (Dittmann 1968). Labeled compounds were located by autoradiography or radioscanning using a Betascan-I (Berthold, Wildbad/F.R.G.), scraped off the plates, and measured by scintillation counting in a Tricarb 3380 (Packard, Frankfurt/F.R.G.).

Electron Microscopy

The pellet of washed mitochondrial was fixed according to Franke et al. (1989) and embedded in ERL (Spurr 1969). The ultrathin sections were double stained in uranylacetate and lead citrate (Reynolds 1963) and observed in a Siemens Elmiskope IA.

Other Methods and Chemicals

Polyacrylamide disc-electrophoresis was carried out according to Hartmann et al. (1973). Protein was determined as given by Lowry et al. (1951). [1,5-14C] citrate was purchased from Buchler/Amersham (Braunschweig/F.R.G.). All chemicals were of an analytical grade.

Results

Alteration of GDH Activity and Isoenzyme Pattern

Detached young pea shoots floated on tap water show a considerable increase in GDH activity within 12–15 h (Fig. 1 A). The increase in activity is characterized by a pronounced lag-phase followed by a sud-