Glutamate Dehydrogenase of *Pisum sativum:* Heat-Dependent Interconversion of the Multiple Forms

Adelheid Ehmke, Heinz-Walter Scheid, and Thomas Hartmann

Institut für Pharmazeutische Biologie der Technischen Universität Braunschweig, Mendelssohnstraße 1, D-3300 Braunschweig

Z. Naturforsch. **39c**, 257–260 (1984); received November 22, 1983

Pisum sativum, Glutamate Dehydrogenase, Multiple Forms, Heat-Dependent Interconversion, Conformational Variants

Purified NAD-dependent glutamate dehydrogenase (EC 1.4.1.2) from pea seeds shows a pattern of seven catalytically active molecular forms. The individual forms display different heat stabilities. During incubation at 70 to 75 °C in the presence of protective agents (NADH, Ca^{2+}, DTE) the more heat labile forms are converted into the most stable form. This result presents direct evidence that the multiple forms of pea glutamate dehydrogenase represent conformational variants of a single protein species.

Introduction

The existence of electrophoretically distinct multiple forms of glutamate dehydrogenase (L-glutamate: NAD^+ oxidoreductase, deaminating; EC 1.4.1.2) is well established for a number of higher plants [1–5]. In pea seeds and seedlings (*Pisum sativum* var. Presto) GDH constitutes a pattern of seven catalytically active bands which remain stable during purification [6] and which are located within the mitochondria [7]. It has been repeatedly suggested that the molecular forms could represent conformational variants of a single protein:

- Denaturation of partially purified enzyme with urea and subsequent renaturation revealed one activity band [4].
- The seven multiple forms of homogeneously pure GDH have identical oligomere and subunit molecular weights as determined by sedimentation equilibrium measurements and SDS gel electrophoresis, respectively [6].
- The multiple forms are immunoidentical [8].

Here we present direct evidence in favour of this suggestion; we report on the heat-dependent interconversion of the multiple forms.

Abbreviations: GDH, glutamate dehydrogenase; DTE, dithioerythritol; PMSF, phenylmethylsulfonylfluoride; BSA, bovine serum albumin.

Reprint requests to Prof. Dr. T. Hartmann.

0341-0382/84/0300-0257 $ 01.30/0

Materials and Methods

Enzyme preparation and assay

Seeds of *Pisum sativum* L. var. Presto (van Waveren Pflanzenzucht, Rosdorf) were used as enzyme source. The purification procedure for GDH was the same as described in ref. [6]. This method include the following purification steps: (1) Acid precipitation, (2) Blue-Sepharose chromatography, (3) Sepharose 6B chromatography, (4) DEAE-Cellulose chromatography. Experiments were peformed with partially purified enzyme (Blue-Sepharose eluate; spec. activity about 800 nkat/mg protein) and homogeneously pure enzyme (DEAE-cellulose eluate, spec. activity about 7000 nkat/mg protein). NADH-dependent GDH activity was assayed photometrically at 366 nm and 25 °C according to ref. [6].

Heat treatment

Test tubes (10 ml) containing 1 ml enzyme solution in 0.05 M Tris-HCl, pH 7.5 (initial activity 35–170 nkat) and additives as indicated, were incubated in a waterbath at 70° or 75 °C (± 1 °C). Aliquots (100 µl) were removed at the time intervals indicated, cooled down in an icebath and used immediately for activity assay or gel electrophoresis. Heat treatments were performed with partially pure and homogeneously pure enzyme (see above). Apart from a somewhat higher heat lability of the pure enzyme, both preparations behave identical.

Electrophoresis

Polyacrylamide gel electrophoresis was performed according to Maurer [9] using the separation system

1a with 6.0 or 6.5% acrylamide. Activity staining was performed as described in [5] and protein staining with Coomassie brillant blue as in [10]. For separation of bands 7 and 1–6, gels were cut in slices. GDH was eluted with 0.05 M Tris-HCl buffer, pH 7.5, and concentrated in an Amicon ultrafiltration cell.

Chemicals

Purine and pyridine nucleotides (grade I) were from Boehringer (Mannheim). All other chemicals were of analytical grade. Blue-sepharose was obtained from Pharmacia Fine Chemicals (Freiburg).

Results and Discussion

Gelelectrophoresis of purified pea GDH shows seven catalytically active bands. According to the recommendations by IUPAC [11] these bands are marked 1 through 7, number 7 exhibiting the least mobility toward the anode. The intensity of the seven bands gradually decreased from band 7 to 1 when stained for protein or GDH activity (see Fig. 2). The isoelectric points of the individual bands were shown to be in the range of pH 5.4 (band 1) to pH 6.4 (band 7). Under normal conditions the multiple pattern remains stable throughout the GDH purification procedure.

The multiple pattern is not altered by the enzyme extraction method applied, or prolonged incubation of crude extracts with or without proteinase inhibitor (PMSF). Essentially identical multiple patterns were obtained from isolated mitochondria (pea seedlings) and crude cell homogenates [7]. Thus any kind of artificial covalent modification (action of proteinase, deamidation etc.) during the course of enzyme preparation appears unlikely. Furthermore incubation of GDH preparations with glucosidase and acid phosphatase revealed no indication for posttranslational modification by glycosidation or phosphorylation.

On the other hand, during prolonged storage (several months) of the purified enzyme or repeated freezing and thawing we observed some alteration of the spectrum: Bands 1, 2 and 3 tended to vanish, whereas band 7 seemed to increase in protein content and activity as judged from electrophoretic analysis. Similar effects were observed when enzyme preparations were incubated at elevated temperatures. Purified GDH was stable at temperatures up to 50 °C but was rapidly inactivated above 70 °C. During temperature inactivation the fast migrating bands (1 to 5) gradually disappeared whereas band 7 remained stable. This phenomenon was studied in more detail.

Temperature inactivation is reduced in the presence of protective agents such as pyridine nucleotides and DTE (Table I). The combination of NADH with Ca^{2+} or with DTE was found to be most efficient (Fig. 1). Purine nucleotides and the

Table I. Heat inactivation of GDH. Incubations were performed at 75 °C for 30 min in the presence of potential protective agents as indicated.

Additive (final concentration)	% of initial GDH activity
None	11
BSA (1 mg/ml)	14
DTE (2 mmol/l)	50
Glycerol (50 µl/ml)	16
NADH (2 mmol/l)	55
NAD^+ (2 mmol/l)	50
ATP (2 mmol/l)	4
ADP (2 mmol/l)	6
Ca^{2+} (1 mmol/l)	18
2-Oxoglutarate (10 mmol/l)	10
L-Glutamate (10 mmol/l)	12

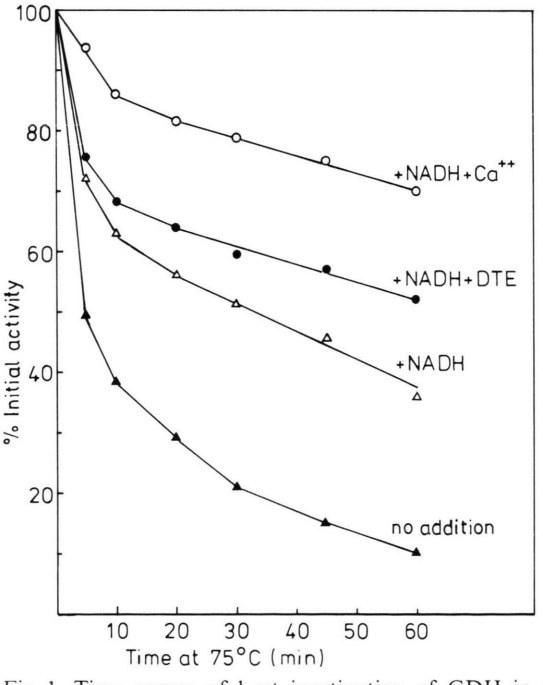

Fig. 1. Time course of heat inactivation of GDH in the presence of protective agents (final concentration 1 mmol/l each).

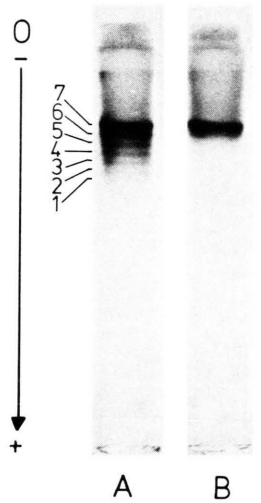

Fig. 2. Polyacrylamide gel electrophoresis of purified GDH (6.5% gel). A: Control; B: After incubation at 75 °C for 30 min in the presence of NADH and Ca^{2+} (1 mmol/l each).

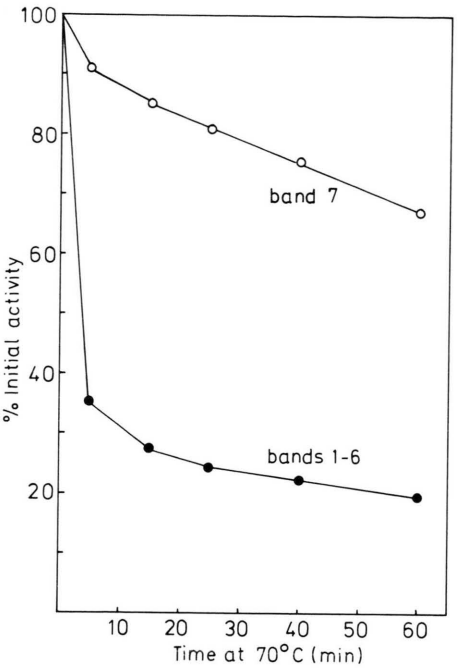

Fig. 3. Time course of heat inactivation of isolated GDH bands 1–6 and band 7. Incubation was performed without addition of protective agents.

substrates 2-oxoglutarate and L-glutamate were ineffective. The loss of GDH activity was about 20% of initial activity when incubated at 75 °C for 30 min in the presence of NADH + Ca^{2+} (Fig. 1). This loss of activity was accompanied by a disappearance of the bands 1 to 6 (Fig. 2). Since these bands account for about 50% of initial GDH activity, the activity of band 7 must have been increased during the heat treatment.

This result indicates that the fast migrating bands 1 to 6 could be converted into band 7. To prove this unequivocally the following experiment was performed. A mixture of bands 1 to 6 and band 7 were isolated from gels and the two preparations were incubated at 70 °C. As shown in Fig. 3 bands 1 to 6 were considerably more temperature labile than band 7, at least during the initial 10 minutes. Gelelectrophoretic analysis revealed that during this period bands 1 to 6 had largely disappeared whereas band 7 concomitantly appeared (Fig. 4). The isolated band 7 remained qualitatively unaltered.

The existence of multiple molecular forms is a common feature of a number of enzymes. They may be caused by at least three different mechanisms:

1) Genetic multiplicity as in the case of true isoenzymes such as lactate dehydrogenase [12] and aldolase [13].

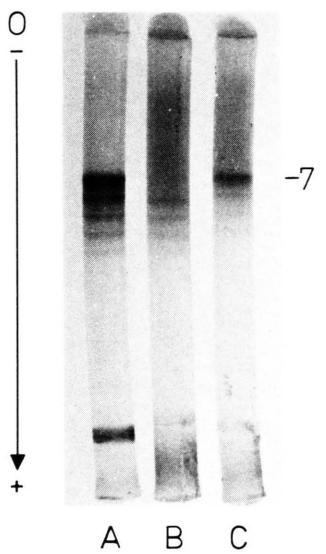

Fig. 4. Polyacrylamide gel electrophoresis of purified GDH (6.0% gel). A: Control (complete pattern); B: Isolated GDH bands 1–6; C: as B but after incubation at 70 °C for 20 min in the presence of NADH and Ca^{2+} (1 mmol/l each).

2) Posttranslational modification by proteolytic activities [14], or phosphorylation/dephosphorylation [15–18] or deamidation [19].

3) Conformational variants of a single protein species assuming multiple, metastable conformational states [20], as shown for the mitochondrial malate dehydrogenase [21, 22] and ascarid enolase [23].

The data reported here are consistent with the view that the multiple forms of GDH are metastable conformational variants which differ in their response to heat inactivation and can be partially converted into the most thermostable variant. The observed stability of the different forms throughout the purification can be explained by relative high activation energy barriers [20]. During incubation at temperatures above 70 °C this energy barrier is overcome. The protecting agents, especially NADH combined with Ca^{2+}, a known activator of plant GDH [6, 24–28], seem to stabilize the active site thus preventing denaturation.

Acknowledgements

We would like to thank van Waveren-Pflanzenzucht (Rosdorf) for supplying us with pea seeds and Mrs. I. Rahaus for technical assistance.

[1] D. A. Thurman, C. Palin, and M. V. Laycock, Nature **207,** 192–194 (1965).
[2] S. B. Yue, Plant Physiol. **44,** 453–457 (1969).
[3] K. Chou and W. E. Splittstoesser, Plant Physiol. **49,** 550–554 (1972).
[4] E. Pahlich, Planta **104,** 78–88 (1972).
[5] T. Hartmann, M. Nagel, and H.-J. Ilert, Planta **111,** 119–128 (1973).
[6] H.-W. Scheid, A. Ehmke, and T. Hartmann, Z. Naturforsch. **35c,** 213–221 (1980).
[7] W. Nauen and T. Hartmann, Planta **148,** 7–16 (1980).
[8] E. Pahlich, W. Ott, and B. Schad, J. Exp. Bot. **31,** 419–423 (1980).
[9] H. R. Maurer, Disc Electrophoresis 2nd edn., 222 (1971).
[10] R. F. Peterson, Methods in Enzymology **25B,** 178–182 (1972).
[11] JUPAC-IUB Commission on Biochemical Nomenclature (CBN) Eur. J. Biochem. **82,** 1–3 (1978).
[12] C. L. Markert, Ann. N. Y. Acad. Sci. **151,** 14–40 (1968).
[13] W. J. Rutter, T. Rajkumar, E. Penholt, and M. Kochman, Ann. N.Y. Acad. Sci. **151,** 616–626 (1968).
[14] B. L. Horecker, in: Isoenzymes (C. L. Markert ed.), Vol. 1, pp. 11–38, Academic Press, New York 1975.
[15] D. P. Baccanari and Sungman Cha, J. Biol. Chem. **248,** 15–24 (1973).
[16] D. R. Randall, M. Williams, and B. J. Rapp, Arch. Biochem. Biophys. **207,** 437–444 (1981).
[17] R. Hemmings, J Biol. Chem. **253,** 5255–5258 (1978).
[18] R. Hemmings, J. Biol. Chem. **255,** 7925–7932 (1980).
[19] J. A. Williams and R. A. John, Biochem. J. **177,** 121–127 (1979).
[20] C. J. Epstein and A. N. Schechter, Ann. N.Y. Acad. Sci. **151,** 85–101 (1968).
[21] G. B. Kitto, P. M. Wasserman, and N. O. Kaplan, Proc. Nat. Acad. Sci. **56,** 578–585 (1966).
[22] G. B. Kitto, F. E. Stolzenbach, and N. O. Kaplan, Biochem. Biophys. Res. Commun. **38,** 31–39 (1970).
[23] S. F. Gockel and H. G. Lebherz, J. Biol. Chem. **256,** 3877–3883 (1981).
[24] K. W. Joy, Phytochemistry **12,** 1031–1040 (1973).
[25] A. Ehmke and T. Hartmann, Phytochemistry **15,** 1611–1617 (1976).
[26] A. Ehmke and T. Hartmann, Phytochemistry **17,** 637–641 (1978).
[27] W. J. Garland and D. T. Dennis, Arch. Biochem. Biophys. **182,** 614–625 (1977).
[28] R. Kindt, E. Pahlich, and I. Rasched, Eur. J. Biochem. **112,** 533–540 (1980).