Plant Physiology and Biochemistry 83 (2014) 225–231

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Glutamate dehydrogenase isoenzyme 3 (GDH3) of *Arabidopsis thaliana* is less thermostable than GDH1 and GDH2 isoenzymes

Laura Marchi [a], Eugenia Polverini [b], Francesca Degola [a], Enrico Baruffini [a], Francesco Maria Restivo [a, *]

[a] Dipartimento di Bioscienze, Università di Parma, Parco Area delle Scienze 11/A, 43124 Parma, Italy
[b] Dipartimento di Fisica e Scienze della Terra, Università di Parma, Parco Area delle Scienze 7/A, 43124 Parma, Italy

ARTICLE INFO

Article history:
Received 20 June 2014
Accepted 5 August 2014
Available online 19 August 2014

Keywords:
Glutamate dehydrogenase
Isoenzymes
Subunits
Thermal stability

ABSTRACT

NAD(H)-glutamate dehydrogenase (GDH; EC 1.4.1.2) is an abundant and ubiquitous enzyme that may exist in different isoenzymic forms. Variation in the composition of the GDH isoenzyme pattern is observed during plant development and specific cell, tissue and organ localization of the different isoforms have been reported. However, the mechanisms involved in the regulation of the isoenzymatic pattern are still obscure. Regulation may be exerted at several levels, i.e. at the level of transcription and translation of the relevant genes, but also when the enzyme is assembled to originate the catalytically active form of the protein. In *Arabidopsis thaliana*, three genes (*GDH1*, *GDH2* and *GDH3*) encode three different GDH subunits (β, α and γ) that randomly associate to form a complex array of homo- and hetero-hexamers. In order to asses if the different *Arabidopsis* GDH isoforms may display different structural properties we have investigated their thermal stability. In particular the stability of GDH1 and GDH3 isoenzymes was studied using site-directed mutagenesis in a heterologous yeast expression system. It was established that the carboxyl terminus of the GDH subunit is involved in the stabilization of the oligomeric structure of the enzyme.

1. Introduction

In higher plants several genes encoding NAD(H) dependent glutamate dehydrogenase (GDH; EC 1.4.1.2) have been described (Dubois et al., 2003). Plant GDH is an abundant and ubiquitous mitochondrial enzyme that catalyzes the reversible amination of glutamate and the presence of the enzyme in the different organs/tissue/cells is regulated by developmental and nutritional cues that are not yet fully understood. In *Arabidopsis thaliana* three GDH genes, *GDH1*, *GDH2* and *GDH3*, encode the β, α and γ subunits of the enzyme (Dubois et al., 2003; Pavesi et al., 2000; Restivo, 2004; Turano et al., 1997; Yamada et al., 2003). These three subunits can randomly associate to form distinct hetero- or homo-hexamers (GDH1 = 6β; GDH2 = 6α; GDH3 = 6γ) depending either on the organ examined or the physiological status of the plant (Fontaine et al., 2006; Igarashi et al., 2009; Miyashita and Good, 2008; Restivo, 2004; Skopelitis et al., 2007; Turano et al., 1997; Watanabe et al., 2007). In particular, it was shown that the activity of the GDH3 isoform is specifically localized in the roots of *Arabidopsis* mature plants and in their immature stamens (Fontaine et al., 2013, 2012; Marchi et al., 2013). Moreover, nitrogen starvation could induce both the homo- (6γ) and hetero- (6 β/γ) isoforms in the roots of young plants hydroponically cultivated (Marchi et al., 2013). From the data of kinetin treatments of plants, it was then concluded that the C/N status and the cytokinin signaling pathways are involved in the process of GDH3 activation in *Arabidopsis* (Marchi et al., 2013). By the use of single (*gdh1* and *gdh2*) and double (*gdh1-2*) GDH mutants impaired in the expression of the β and/or α subunit it was previously evidenced that regulation of transcription of the relevant genes is involved in the determination of the isoenzymatic pattern observed in the different organs of *A. thaliana* (Fontaine et al., 2013). It was also speculated that compensatory mechanisms, involving reciprocal regulation among *GDH1*, *GDH2* and *GDH3* modulate the overall GDH activity and determine the specificity of the isoenzyme profile (Fontaine et al., 2013, 2012). However regulation of GDH isoforms activity may also depend on different (i.e. post-transcriptional) regulatory mechanisms. A paradigmatic example is provided by human GDH

Abbreviations: GDH, NAD(H)-dependent glutamate dehydrogenase.

* Corresponding author. Tel.: +39 0521 905603; fax: +39 0521 905604.
E-mail addresses: francescomaria.restivo@unipr.it, restivo@unipr.it (F.M. Restivo).

http://dx.doi.org/10.1016/j.plaphy.2014.08.003
0981-9428/

(hGDH) that, as in plants, has an hexameric structure (Shashidharan et al., 1997). The hGDH subunits are encoded by two genes, hGDH1 and hGDH2, giving rise to different isoforms with tissue specific localization and different thermal stability (Shashidharan and Plaitakis, 2014). In addition several GDH isoproteins differing in molecular mass and isoelectric point have been detected in human brain (Plaitakis et al., 2000).

hGDH is subject to a complex allosteric regulation by various ligands such as ADP, GTP and L-leucine depending also (but not exclusively) on the presence of a peculiar structure, named "antenna", not present in plants and bacteria GDH (Li et al., 2012). Moreover a different type of interaction at the interface of hGDH enzyme subunits has been suggested to provide a novel allosteric site for L-leucine resulting in a fine-tuned mechanism to control enzyme activity (see the review reported above and references therein). In plants, no evidence of allosteric regulation by ADP, GTP or leucine has been until now reported. However, in bacteria, whose GDH also is deprived of an antenna like structure, examples of homo- and hetero-tropic allosteric enzyme regulation have been reported [see (Tomita et al., 2010) and references therein]. Interestingly, plant GDH amino acid sequences are more similar to the archaeobacterial counterparts than to the eukaryotic GDH (Pavesi et al., 2000; Syntichaki et al., 1996). Accordingly an increased thermo stability of plant GDHs has been observed when compared to that of lower and higher eukaryotes (Pavesi et al., 2000; Syntichaki et al., 1996). Thus it may not be excluded that plants may have also evolved, in addition to transcriptional control of gene expression, a post-translational mechanism (albeit different from those previously described for hGDH) to regulate GDH enzyme activity. Moreover it has been speculated that GDH may require a fine tuning and rapidly responding mechanism (and hence at the post-translational level) to cope with transitions from stress to post-stress recovery (Limami et al., 2014, 2008). For instance, GDH subunits assembling to form a hexamer and its stability in a specific cell environment may be crucial for enzyme activity and, as such, the possible target for a regulatory step. In this sense, even if a multiplicity of experimental data concerning the activity of various effectors on the modification of GDH isoenzyme pattern have been reported in the literature, the biochemical mechanisms regulating the process of GDH subunits isomerization in plants have been scarcely considered, with few exceptions (Osuji et al., 2003, 1999, 1997), and are not yet fully understood.

In the present work the thermal stability of the different *Arabidopsis* GDH isoforms was investigated in order to determine the possible presence of a structure–function relationship of the protein.

2. Materials and methods

2.1. Plant material and growth conditions

WT, single (*gdh1* or *gdh2*) and double (*gdh1-2*) GDH mutants of *A. thaliana* (ecotype Columbia) were grown in vitro conditions. GDH single mutants were obtained from the *Arabidopsis* Stock Center and have been described elsewhere (Fontaine et al., 2006); double mutants were obtained by crossing single mutants. Prior to sowing, seeds were surface sterilized for 20 min in a diluted bleach solution (final concentration = 0.5% v/v Sodium Hypochlorite) containing 0.1% (v/v) Tween 20. The seeds were then washed three times with sterile demineralized water and stratified for two days in the dark at 4 °C. Stratified seeds were germinated in Petri dishes on 1/2 Murashige-Skoog (MS) agar medium; (MS salts; Duchefa; #M0222, Haarlem, The Netherlands) containing 2% (w/v) sucrose and solidified with 0.8% (w/v) agar) and grown for 7 days at 25 °C with a 16 h/8 h photoperiod (150 μmol photons m^{-2} s^{-1}). To induce GDH3 activity in plants, *gdh2* and *gdh1-2* double mutants were transferred to N-starvation conditions as previously described (Marchi et al., 2013): seven day old plants were removed from the solid medium and transferred to Petri dishes filled with 10 ml of 1/5 diluted MS medium containing 1% sucrose (adaptation to liquid culture condition) without shaking. After 2 days plants were quickly washed with sterile demineralized water and transferred to the N-starvation medium (1/5 MS salts deprived of KNO_3 and NH_4NO_3) for 5 days. For sample analysis, whole plants (for WT and *gdh1* mutant) or excised roots (for *gdh2* and *gdh1-2* mutants) were collected, blotted dry on filter paper, dipped in liquid N and stored at −80 °C. *Nicotiana plumbaginifolia* seeds belonging to a GDHA silenced mutant line (Fontaine et al., 2006) were processed, germinated and grown in the same in vitro conditions described above for *A. thaliana*. Protein extracts of this mutant line were used for reconstruction experiments in which the thermostability of *A. thaliana* and *N. plumbaginifolia* GDH were compared (see Supplementary Fig. S1)

2.2. Protein extraction and in gel GDH activity staining

Proteins were extracted from different tissues as described previously (Fontaine et al., 2006; Restivo, 2004). Briefly, 200 mg of plant tissue was homogenized in 200 μl of extraction buffer composed of 100 mM Tricine (pH 8.0), 10 mM $MgSO_4$, 0.2% (v/v) β-mercaptoethanol, 0.5 mM PMSF, 40 mM $CaCl_2$, 0.5% (w/v) polyvinylpyrrolidone, 1 mM EDTA and 0.05% (v/v) Triton X-100 in a 1.5 mL Eppendorf tube using a micro-pestle. After two rounds of centrifugation at 15,000 × *g* for 20 min at 4 °C, the resulting supernatant (10 μl) was used for GDH *in gel* activity detection. *In gel* NAD-GDH activity detection was performed as described by Loulakakis and Roubelakis-Angelakis (1990), with minor modifications. The GDH activity staining solution, containing 100 mM Tris–HCl (pH 8.8), 53 mM sodium glutamate, 0.7 mM NAD, 0.03 mM phenazine methosulphate and 0.3 mM Nitro Blue Tetrazolium (NBT) was supplemented with agarose (BioRad, Hercules, CA, USA) at a final concentration of 0.4% (w/v) and poured onto the gel. Enzyme activity staining was performed at 37 °C in the dark and stopped by replacing the staining solution with distilled water. Photographs of the gel were taken with a Kodak EDAS120 digital camera (Eastman Kodak Company, Rochester, NY, USA) and the activity of the different GDH isoenzymes was quantified by the 1D image analysis software provided by the manufacturer. Both PAGE and *in gel* GDH activity detection were performed at least in triplicate with different plants for each experiment. Comparable results were obtained in each replicate.

2.3. GDH mutant protein construction and heterologous expression in yeast

Total RNA was extracted from in vitro grown plants using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Two μg of RNA were then reverse-transcribed using the ImProm-II® reverse transcription kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. The cloning site *Bam*HI was added by PCR to the 5′ end of the *GDH1* and *GDH3* cDNAs using specific primers (Supplementary Table S1). Mutant proteins were obtained by introducing the required amino acid substitutions (A411S for GDH1 and I397V, I406L and S411A for GDH3) in *GDH1* and *GDH3* cDNA sequences by PCR, using specific primers (Supplementary Table S1). The amplification products were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA), sequenced and sub-cloned in the *Bam*HI and *Eco*RI or *Sal*I sites of the pYEX-BX vector (Clontech Laboratories, Palo Alto, CA, USA). The resulting vectors were then transferred from *Escherichia*