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SHORT COMMUNICATION

Involvement of non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase in response to oxidative stress

Diego M. Bustos[a], Claudia A. Bustamante[a], Alberto A. Iglesias[b,*]

[a]*Instituto Tecnológico de Chascomùs (IIB-INTECH), Cam. Circ. Lag. km 6, CC 164, B7130IWA Chascomùs, Argentina*
[b]*Laborarotio de Enzimología Molecular, Facultad de Bioquímica y Ciencias Biológicas, UNL, Paraje "El Pozo" CC 242, S3000ZAA Santa Fe, Argentina*

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Summary

Glyceraldehyde-3-phosphate dehydrogenases catalyze key steps in energy and reducing power partitioning in cells of higher plants. Because non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (NP-Ga3PDHase) is involved in the production of reductive power (NADPH) in the cytosol, its behavior under oxidative stress conditions was analyzed. The specific activity of the enzyme was found to increase up to 2-fold after oxidative conditions imposed by methylviologen in wheat and maize seedlings. Under moderate oxidant concentration, lack of mRNA induction was observed. The increase in specific activity would thus be a consequence of a significant stability of NP-Ga3PDHase. Our results suggest that the enzyme could be modified by oxidation of cysteine residues, but formation of disulfide bridges is dependent on levels of divalent cations and 14-3-3 proteins. The latter differential effect could be critical to relatively maintain energy and reductant levels in the cytoplasm of plant cells under oxidative stress.

Introduction

Reactive oxygen species (ROS) cause damage to proteins, lipids, and nucleic acids and thereby compromise cell viability. An exhaustive characterization of the yeast H_2O_2-stimulon has demonstrated that oxidative stress induces responses at the systemic and local levels, with a remarkable change occurring in carbohydrate metabolism (Godon et al., 1998). In this organism, NAD-dependent glyceraldehyde-3P (Ga3P) dehydrogenase isoenzymes are inactivated after oxidative stress, causing a slow-down in glycolysis. Thus, glucose is diverted to the pentose-phosphate pathway to generate additional NADPH, a main cofactor for antioxidant enzymes

*Corresponding author. Tel./fax: +54 342 4575216x217.
E-mail address: iglesias@fbcb.unl.edu.ar (A.A. Iglesias).

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(Godon et al., 1998). In plants, H_2O_2 has also been recognized as a second messenger for signals triggered by ROS (Yang et al., 2006), but the occurrence of changes in carbon metabolism in the cytosol coping with the stress conditions was not characterized.

Plant cells, distinctively from other eukaryotes, have two enzymes with Ga3P dehydrogenase activity in the cytosol. One is the above-mentioned NAD-dependent Ga3P dehydrogenase, a typical enzyme involved in glycolysis that is found in all other organisms. The odd enzyme in plants is NADP-dependent, non-phosphorylating, Ga3P dehydrogenase (D-glyceraldehyde-3-phosphate:$NADP^+$ oxidoreductase; EC 1.2.1.9; non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (NP-Ga3PDHase)) that catalyzes the essentially irreversible reaction

$$\text{D-Ga3P} + \text{NADP}^+ + \text{H}_2\text{O} \rightarrow \text{3PGA} + \text{NADPH} + 2\text{H}^+.$$

The enzyme was first described in photosynthetic cells (Arnon et al., 1954; Rosenberg and Arnon, 1955) and then purified and characterized from a number of vascular plants and green algae. It has been determined that a single gene (*gapn*) codes for NP-Ga3PDHase and that the enzyme is modified by post-translational phosphorylation in non-photosynthetic plant cells (Bustos and Iglesias, 2002). An early proposal (Kelly and Gibbs, 1973) states the functional role of NP-Ga3PDHase as metabolizing photosynthetically generated NADPH. In such a scenario, Ga3P produced in the Calvin cycle is transported (via the chloroplast phosphate-translocator) to the cytosol. It follows that NP-Ga3PDHase reduces $NADP^+$ to NADPH coupled to oxidation of Ga3P to 3-PGA, which is then transported back into the plastid. The overall process results in a triose-phosphate-driven NADPH shuttle mechanism between the chloroplast and the cytosol. It has been proposed that under conditions of high photosynthetic activity, the pentose-phosphate pathway is inhibited and the activity of NP-Ga3PDHase is a potentially relevant source of cytosolic NADPH (Anderson, 1986; Habenicht, 1997). Less clear is the role played by the enzyme in non-photosynthetic tissues of plants.

Since NP-Ga3PDHase produces NADPH in the cytosol, the study of how it is affected by oxidative conditions is relevant to analyze the capacity of plant cells in coping with stressful environments. In this report, we found that the activity of the enzyme is relatively increased in wheat and maize seedlings exposed to the chemical oxidant methylviologen (MV), and the relevance of this in the generation of cytosolic NADPH is discussed in the context of the stressed plant cell metabolism.

Materials and methods

Materials

Diamide, MV, D-Ga3P, fructose-1,6-bisphosphate, aldolase, $NADP^+$, NBT, BCIP, and H_2O_2 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Wheat (*Triticum aestivum*) and maize (*Zea mays*) plants were grown for 7–8 d in a growth chamber under a 16 h light/8 h dark regime, with a light intensity of 200 $\mu mol\,m^{-2}\,s^{-1}$, 75% relative humidity, and day/night temperatures of 21/19 °C.

Oxidative treatments

Chemical stress on plant tissue was imposed as described in Palatnik et al. (1997), by spraying 40 seedlings with 20 mL of a 0.05% (v/v) Tween 20 solution containing different concentrations of MV or with an equal volume of distilled water. Plants were then illuminated (200 $\mu mol\,m^{-2}\,s^{-1}$) for 8 h before sampling.

Oxidative treatments on purified NP-Ga3PDHase were performed in the dark at 30 °C, by incubating the enzyme with the stated concentrations of H_2O_2 during 10 min. Incubation medium contained 50 mM Tricine–NaOH (pH 8.5) and 10% glycerol, with further additions specified in each case. Reversion of the oxidative modification was carried out by addition of 20 mM 2-mercaptoethanol on the corresponding medium. At different times, aliquots (10 μL) were withdrawn from the incubation medium and assayed for activity as described below.

Protein extraction

Five grams (fresh weight) of wheat and maize seedlings was homogenized in a medium containing 20 mM Tris–HCl (pH 7.0), 1 mM EDTA, and 20 mM 2-mercaptoethanol. Extracts were clarified by centrifugation (15 min at 10,000 *g*) and protein concentration was determined by the Bradford method (Bradford, 1976), using BSA as standard.

Purification of NP-Ga3PDHase, kinetic measurements, and enzyme inhibition

The NP-Ga3PDHase was partially purified from wheat and maize seedlings as described previously (Bustos and Iglesias, 2003). Enzyme activity was assayed spectrophotometrically by monitoring NADPH generation at 340 nm as described previously (Bustos and Iglesias, 2003). Assays were performed at 30 °C in a medium (1 mL final volume) containing 50 mM Tricine–NaOH (pH 8.5), 0.11 mM $NADP^+$, and 1.2 mM D-Ga3P.

Nucleic acid extraction and RT-PCR

Retrotranscription was performed using 2 μg (as quantified spectrophotometrically) of total RNA from wheat or maize extracted with guanidine isotiocyanate

phenol–chloroform method (Sambrook and Russell, 2001). Primers T3 (5′-GCGGGAATTCCAAATCTCACG-3′, underline corresponds to an *Eco*RI site) and 25Sr (5′-GTTAGTCGATCCTAAGGGTC-3′) were used in the retrotranscription of *gapn* and ribosomal RNA 25S, respectively, with the MLM-retrotranscriptase (Promega, Madison, WI, USA) following the manufacturer's protocol. After the reaction, cDNA amplification was made with primers T2 and 25Sf (5′-CCKGACATGAGGATCGCATGGG-3′ and 5′-CCTGATGCGGTTATGAGTAC-3′, respectively) every 5 cycles. The cDNA was transferred to nylon membranes (Hybond N^+, Amersham) and hybridization performed overnight with *gapn* ^{32}P-labeled as probe.

SDS-PAGE, protein gel blot analysis, and ELISA

Electrophoresis under denaturing conditions (SDS-PAGE) was performed in 10% gels (Laemmli, 1970). Immunodetection was achieved by the alkaline phosphatase protocol, with NBT and BCIP as substrates and rabbit antibodies against the leaf celery NP-Ga3PDHase (Bustos and Iglesias, 2003). ELISA experiments were performed in a BioRad ELISA plate reader, with microwells Nunc Maxi-Sorp Immuno Plate utilizing 1, 3, 5, 10, and 20 μg of antigen (clarified wheat and maize protein extracts) and secondary antibody conjugated with alkaline phosphatase.

Results and discussion

Effect of MV treatment on NP-Ga3PDHase activity and expression

The presence of NP-Ga3PDHase in plant cells establishes a potential main pathway to produce reducing power from triose-phosphate in the cytosol (Iglesias, 1990; Bustos and Iglesias, 2003). We analyzed levels of the NP-Ga3PDHase in 8-day-old seedlings of wheat and maize sprayed with increasing concentrations of MV. After 8 h of treatment, necrotic spots became evident at 1 and 10 μM of MV in maize and wheat, respectively. Under these conditions, NP-Ga3PDHase-specific activity increased by 1.5- to 2-fold in extracts from wheat and maize seedlings (Figure 1). Relative activity of the enzyme was increased under different conditions, with maximal values occurring at 1 μM in wheat (Figure 1A) and at 0.1 μM in maize (Figure 1B, see Supplementary material). Results suggest that NP-Ga3PDHase remains as an active enzyme, generating NADPH under oxidative stress, even when other proteins become inactive or degraded.

Figure 2 shows the levels of mRNA coding for NP-Ga3PDHase in extracts from control and treated plants of wheat (Figure 2A) and maize (Figure 2B, see Supplementary material). As shown, in both plant species levels of the enzyme transcripts were found practically unaltered at low and moderated (0.05–1 μM) MV treatments, while a slight increase was observed for plants stressed with 10 μM MV. Figure 2 also shows that when plants were confronted with high levels of MV (100 μM),

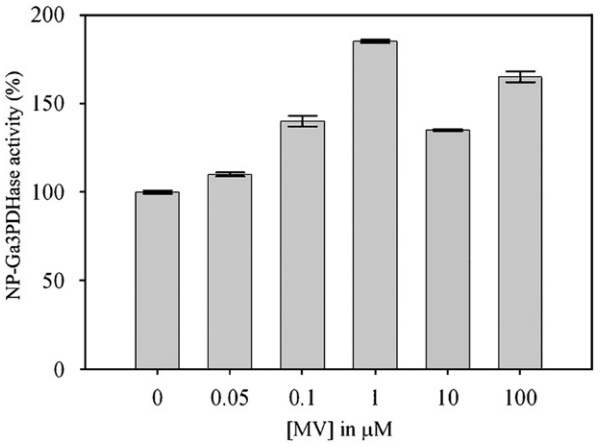

Figure 1. NP-Ga3PDHase activity in wheat (A) and maize (B, see Supplementary material) seedlings treated with MV. Activity of the enzyme was determined in extracts from seedlings sprayed with increasing MV concentrations and relative excess of light (300 μmol photon m^{-2} s^{-1}) during 8 h. Values are expressed as percentage considering 100% activity found in extracts from seedlings treated with no MV. Each value represents the mean of nine independent experiments.

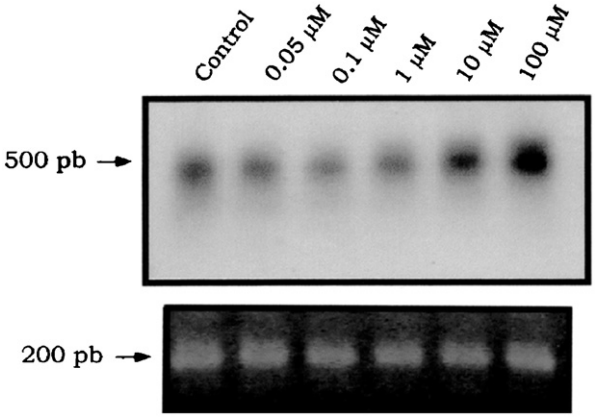

Figure 2. Steady-state levels of mRNA coding for NP-Ga3PDHase in wheat and maize seedlings treated with MV. After semi-quantitative RT-PCR, cDNA products from control or treated seedlings of wheat (A) or maize (B, see Supplementary material) were hybridized with the ^{32}P-labeled *gapn* probe. As a control, a retrotranscribed fragment of 200 pb of the 25S rRNA was stained with ethidium bromide (C, wheat and D in Supplementary material, maize).

accumulation of the mRNA transcribing *gapn* was significantly increased. These results indicate that the expression of NP-Ga3PDHase in plants is not induced by low or moderate but by high treatments with MV. Data suggest that the *gapn* gene has oxidative-responsive elements that operate at severe stress conditions, at least in wheat and maize.

A protein band of approximately 58–60 kDa was detected in immunoblots revealed with NP-Ga3PDHase antiserum, with levels increasing as the concentration of MV was raised (Figure 3). In wheat the increase in NP-Ga3PDHase was evident at 1 μM MV (Figure 3A), whereas in maize higher relative levels of the enzyme were already observed at 0.1 μM MV (Figure 3B, see Supplementary material). The combined analysis of results illustrated by Figures 1–3 suggests that a relative stability of NP-Ga3PDHase mainly accounts for the increase in specific activity observed in treatments with low and medium levels (up to 10 μM) of MV. To reinforce this conclusion, a control was made in experiments reported in Figure 3 with the addition of cycloheximide, to show the absence of *de novo* synthesis of the enzyme during such oxidative conditions (data not shown). It has been reported that unspecific degradation and proteolysis occurring under oxidative stress can reduce protein contents up to 60% (Palatnik et al., 1997; Shigeoka et al., 2002).

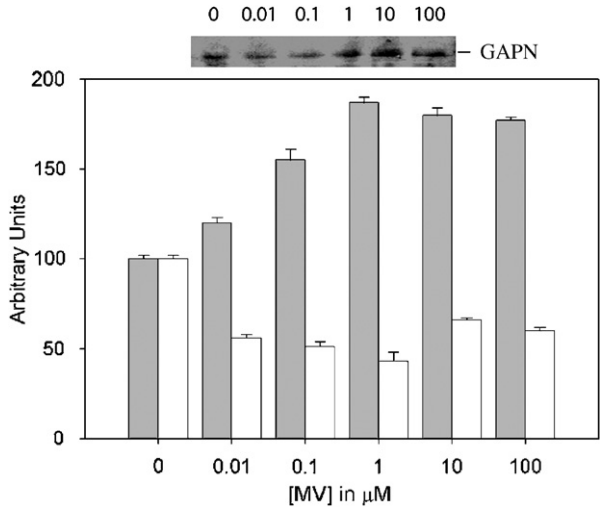

Figure 3. Steady-state levels of NP-Ga3PDHase protein in wheat (A) and maize (B, see Supplementary material) seedlings treated with MV. The amount of NP-Ga3PDHase protein was quantified by ELISA and relative levels are illustrated by gray bars. The white bars represent levels of total protein. In both cases, values are expressed in arbitrary units, considering 100% of the measurement obtained in plants treated with no MV. On the top, the protein gel blot of NP-Ga3PDHase is shown.

We observed a similar decrease (61%) in total protein concentration in both plant seedlings exposed to oxidative stress (Figure 3, see Supplementary material for Fig. 3B). These levels of reduction further support the upraising in NP-Ga3PDHase-specific activity considering that the enzyme remains relatively stable at conditions where other proteins are degraded.

Effect of oxidant compounds on NP-Ga3PDHase stability in wheat and maize seedlings

The apparent stability of NP-Ga3PDHase in plants exposed to oxidative stress supposes a relative low reactivity of the enzyme to oxidant metabolites. However, previous reports demonstrated that the enzyme from photosynthetic organisms (green algae and plant leaves) is sensitive to inactivation by reagents modifying cysteine residues (Iglesias et al., 1987; Iglesias and Losada, 1988; Gómez Casati et al., 2000). To further explore on this molecular approach, we analyzed the effect of reagents oxidizing thiol groups on the enzyme from wheat seedlings. We used partially (about 25-fold) purified NP-Ga3PDHase from this non-photosynthetic plant source to analyze it when it is in a phosphorylated state and forming or not a complex with 14-3-3 proteins (in the absence or in the presence of $MgCl_2$, respectively), which has been demonstrated to produce changes in the kinetic properties of the enzyme (Bustos and Iglesias, 2002, 2003). We analyzed two different thiol-oxidizing reagents: diamide, a compound utilized in the identification of essential cysteine residues (Iglesias et al., 1987), and H_2O_2, a metabolite signaling oxidative conditions in plant cells (Yang et al., 2006).

NP-Ga3PDHase from wheat seedlings was sensitive to inactivation by diamide, which was independent of the presence of $MgCl_2$ in the medium (data not shown). Results are in good agreement with those previously reported for the green algae enzyme (Iglesias et al., 1987), as the wheat seedlings' enzyme lost near 70% of activity after incubation with 5 mM diamide by 30 min. Also, inactivation was protected by substrate Ga3P (with no effect by $NADP^+$) and it was completely reversed by the thiol-reducing agent 2-mercaptoethanol. Results obtained with H_2O_2 were significantly different, as NP-Ga3PDHase was inactivated at different degrees depending on the presence or absence of Mg^{2+} in the medium (Table 1). Thus, in the absence of the divalent cation the enzyme remained 80% active, whereas near 75% of the

Table 1. Effects of hydrogen peroxide on NP-Ga3PDHase enzyme

H_2O_2 (μM)	NP-Ga3PDHase activity (%)					
	None	$NADP^+$	D-Ga3P	Mg^{2+}	Mg^{2+}+$NADP^+$	Mg^{2+}+D-Ga3P
0	100	98.7	99.4	100	100	100
20	90.4	95.5	92.0	35.4	36.9	36.0
100	88.2	86.8	87.8	32.6	31.3	31.7
1000	72.0	73.2	72.4	22.2	24.3	22.5

Treatment with the oxidant was performed as described under Materials and methods, in the absence or in the presence of $NADP^+$ (1.1 mM), Ga3P (1.2 mM), and/or $MgCl_2$ (5 mM). Results are means of three independent experiments.

activity was lost when 5 mM $MgCl_2$ was included in the incubation medium. The substrates ($NADP^+$ and D-Ga3P) have no effect by themselves on the inactivation of the enzyme by H_2O_2 (Table 1). Results suggest that the complex NP-Ga3PD-Hase–14-3-3 was relatively stable to oxidation by H_2O_2, and its dissociation by Mg^{+2} makes the enzyme more susceptible to inactivation. This inactivation was completely irreversible, as no change in residual activity was observed after incubation of the enzyme oxidized by H_2O_2 with 20 mM 2-mercaptoethanol during 30 min (data not shown).

It has been reported that low oxidative conditions generated by H_2O_2 at the modest concentration of 10 μM inhibits thiol-modulated enzymes or those containing essential sulfhydryl groups, this being a major target of H_2O_2 reactivity (Tamoi et al., 1996). However, it has been demonstrated that NP-Ga3PDHase from *Streptococcus mutans* is resistant to high concentrations of H_2O_2, meaning that the cysteine located in the catalytic center is recalcitrant to this specific oxidative agent (Arutyunov and Muronetz, 2003). Our results suggest that the plant enzyme is relatively more sensitive to H_2O_2, but this oxidation is different from that produced by diamide, or at least it is distinctively affected by ligands of the enzyme. Structural studies have shown that NP-Ga3PDHase from plants has two cysteine residues that are absent in the enzyme from bacteria. Interestingly, these two cysteine groups are separated and oriented close enough to form a disulfide bond and could be related with the region that interacts with 14-3-3 proteins (Bustos and Iglesias, 2005). It is tempting to speculate that these residues could account for the different sensitivity of the plant enzyme to oxidative stress. Results suggest that NP-Ga3PDHase could be a target for oxidation by metabolites produced during oxidative stress, but the formation of disulfide bonds in the enzyme triggered by the main intracellular signal (H_2O_2) is relatively low and dependent on levels of divalent cations and 14-3-3 proteins. The enzyme could be relatively protected against inactivation and after remaining active it could be a main producer of NADPH in the cytoplasm of plant cells, thus generating a key metabolite to cope with oxidative stress.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jplph.2007.06.005

References

Anderson LE. Adv Bot Res 1986;12:3–46.
Arnon DI, Rosenberg LL, Whatley FR. Nature 1954;173:1132–4.
Arutyunov DY, Muronetz VI. Biochem Biophys Res Commun 2003;300:149–54.
Bradford MM. Anal Biochem 1976;72:248–54.
Bustos DM, Iglesias AA. FEBS Lett 2002;530:169–73.
Bustos DM, Iglesias AA. Plant Physiol 2003;133:2081–8.
Bustos DM, Iglesias AA. J Mol Graph Model 2005;23:490–502.
Godon C, Lagniel G, Lee J, Buhler J, Kieffer S, Perrot M, et al. J Biol Chem 1998;273:22480–9.
Gómez Casati DF, Sesma JI, Iglesias AA. Plant Sci 2000;154:107–15.
Habenicht A. Biol Chem 1997;378:1413–9.

Iglesias AA. Biochem Educ 1990;18:2–5.
Iglesias AA, Losada M. Arch Biochem Biophys 1988;260: 830–40.
Iglesias AA, Serrano A, Guerrero MG, Losada M. Biochim Biophys Acta 1987;925:1–10.
Kelly GJ, Gibbs M. Plant Physiol 1973;52:1023–30.
Laemmli UK. Nature 1970;227:680–5.
Palatnik JF, Valle EM, Carrillo N. Plant Physiol 1997;115: 1721–7.
Rosenberg LL, Arnon DI. J Biol Chem 1955;21:361–71.
Sambrook J, Russell DW. Molecular cloning. A laboratory manual, third ed. Cold Spring Harbor, NY: Cold Spring Habor Press; 2001.
Shigeoka S, Ishikawa T, Tamoi M, Miyagawa Y, Takeda T, Yabuta Y, et al. J Exp Bot 2002;53:1305–19.
Tamoi M, Ishikawa T, Takeda T, Shigeoka S. Biochem J 1996;316:685–90.
Yang X, Lee WH, Sobott F, Papagrigoriou E, Robinson CV, Grossmann JG, et al. Proc Natl Acad Sci 2006;103: 17237–42.