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A possible role for the chloroplast pyruvate dehydrogenase complex in plant glycolate and glyoxylate metabolism

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ABSTRACT

Glyoxylate is a peroxisomal intermediate of photorespiration, the recycling pathway for 2-phosphoglycolate (2-PG) produced by the oxygenase activity of Rubisco. Under hot and dry growth conditions, photorespiratory intermediates can accumulate and must be detoxified by alternative pathways, including plastidal reactions. Moreover, there is evidence that chloroplasts are capable of actively producing glyoxylate from glycolate. Further metabolic steps are unknown, but probably include a CO_2 release step. Here, we report that CO_2 production from glycolate and glyoxylate in isolated tobacco chloroplasts can be inhibited by pyruvate, but not related compounds. We isolated a protein fraction that was enriched for the chloroplast pyruvate dehydrogenase complex (PDC). The fraction contained a protein complex of several MDa in size that included all predicted subunits of the chloroplast PDC and a so far unidentified HSP93-V/ClpC1 heat shock protein. Glyoxylate competitively inhibited NADH formation from pyruvate in this fraction. The K_m for pyruvate and the K_i for glyoxylate were 330 and 270 μM, respectively. Glyoxylate decarboxylation was also enriched in this fraction and could be in turn inhibited by pyruvate. Based on these data, we suggest that the chloroplast PDC might be part of a pathway for glycolate and/or glyoxylate oxidation in chloroplasts.

1. Introduction

Glyoxylate is a central intermediate of photorespiration, by mass flux the second most important biochemical pathway behind photosynthesis (Sharkey, 1988). Photorespiration is the salvage reaction for 2-phosphoglycolate formed during oxygenation of ribulose-1,5-bisphosphate by Rubisco (Bowes et al., 1971). All Rubisco enzymes catalyze this reaction beside their carboxylation function (Andersson, 2008). Photorespiration starts with dephosphorylation of 2-PG in chloroplasts. Resulting glycolate is exported to the peroxisome, where it is oxidized to glyoxylate and transaminated to glycine. Glycine is transported to mitochondria where two molecules of glycine are converted to one molecule of serine. This reaction includes the release of CO_2 and NH_3 that consume energy and reducing power when refixed. Thus, photorespiration is an energy-consuming pathway that can limit plant productivity when high amounts of 2-PG are formed. Serine is transported back to the peroxisome, where it is deaminated and reduced to glycerate. The pathway ends with phosphorylation of glycerate in the chloroplast. The product 3-phosphoglycerate can be integrated into the Calvin cycle and used for carbohydrate production (Ogren, 1984).

The amount of 2-PG synthesized by Rubisco varies dependent on conditions. High temperatures reduce the specificity of Rubisco for CO_2 over O_2 consume energy and reducing power when refixed (Brooks and Farquhar, 1985; Jordan and Ogren, 1984; Ku and Edwards, 1977). Furthermore, high temperatures often coincide with drought. Closure of stomata under these conditions limits gas exchange and CO_2 availability in the leaf intracellular space. All this can result in high rates of 2-PG formation (Cornic and Briantais, 1991; Lawlor and Fock, 1977). Under these conditions, intermediates of photorespiration can accumulate (Campbell and Ogren, 1990; Srivastava et al., 2011) and might be further metabolized by alternative pathways. It has been suggested that glyoxylate can be directly decarboxylated in peroxisomes to formate if capacities of photorespiration are exhausted (Grodzinski, 1978). If this mechanism is also not efficient enough, glyoxylate can diffuse back into the chloroplast and inhibit Calvin cycle enzymes (Campbell and Ogren, 1990; Chastain and Ogren, 1989). Probably for this reason, chloroplasts contain protective enzymes that can reduce glyoxylate back to glycolate (Allan et al., 2009). However, there is also evidence that chloroplasts contain a pathway that oxidizes glycolate to glyoxylate and further to CO_2 (Frederick et al., 1973; Kebeish et al., 2007). When glyoxylate is fed to isolated

Abbreviations: PDC, pyruvate dehydrogenase complex; TPP, thiamine pyrophosphate; 2D-BN/SDS–PAGE, two dimensional blue native SDS–PAGE.

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chloroplasts, CO_2 release from this compound can be observed (Zelitch, 1972). Enzymes catalyzing the reaction steps of this pathway have not been identified so far.

In this paper, we report evidence that the chloroplast pyruvate dehydrogenase complex (PDC) from tobacco is able to decarboxylate glyoxylate and participates in the plastidal conversion of glycolate to CO_2. In plants, PDCs are present in both mitochondria and chloroplasts. Both complexes consist of several copies each of at least four enzymes, which together convert pyruvate to acetyl-CoA under release of CO_2 and production of NADH (Reid et al., 1977). Whereas the mitochondrial complex mainly provides substrates for the citric acid cycle (Randall et al., 1977), the chloroplast homologue produces precursors for fatty acid biosynthesis (Camp and Randall, 1985). Subunits of the chloroplast PDC were previously predicted based on their homology to their mitochondrial counterparts (Johnston et al., 1997). In the present paper, chloroplast PDC and its subunit composition were analyzed by combination of 2D-BN/SDS–PAGE (Schagger et al., 1994) and mass spectrometry resulting in the identification of novel associated proteins. Furthermore, an estimation of the molecular weight of the native PDC is given.

2. Materials and methods

2.1. Plant material and growth conditions

Leaves from 28–32 days old *Nicotiana tabacum* (*cv PDB6*) plants grown under 14 h illumination and 10 h darkness in the greenhouse were used for chloroplast isolation.

2.2. Chloroplast isolation

15 g of leaf material were homogenized 2 times in three 5 s-intervals in a pre-chilled 500 ml Warring blender and chilled grinding buffer containing 50 mM Hepes–KOH, pH 8.0; 2 mM EDTA; 1 mM $MgCl_2$; 5 mM sodium ascorbate; 0.5% w/v BSA. The homogenate was filtered through a 30 μm nylon mesh and the filtrate was centrifuged at 300*g* for 4 min. The pellet was resuspended in a minimal volume of grinding buffer and 1 ml was loaded on a 10 ml 35% v/v self-forming Percoll gradient, containing 50 mM Hepes–KOH, pH 8.0 and 330 mM sorbitol. After centrifugation for 20 min at 19,000 rpm in a swing-out rotor (SW40Ti, Beckman Coulter, Inc.), intact chloroplasts were isolated from the lower green fraction near to the bottom of the gradient. The supernatant was discarded and the chloroplast fraction was washed with 5 vol SH-buffer (50 mM Hepes–KOH, pH 8.0; 330 mM sorbitol; 1 mM $MgCl_2$). For disruption of chloroplasts, the pellet was resuspended in extraction buffer (50 mM Hepes–KOH, pH 8.0; 5 mM $MgCl_2$; 0.1% v/v Triton X-100). All steps were conducted at 4 °C.

2.3. SDS–PAGE and Western blotting

The electrophoretic steps were conducted in a Mini Protean II cell from Bio-Rad (Hercules, USA). Standard protocols were used for SDS–PAGE and Western blot. As weight scale, the PageRuler Plus prestained protein ladder was loaded onto the gel (Thermo Scientific, Schwerte, Germany). The primary antibodies against catalase and cytochrome c oxidase subunit II were purchased from Agrisera (Vännäs, Sweden) and diluted 1/1000 in TTBS (10 mM Tris–HCl, pH 7.5; 150 mM NaCl, 0.05% v/v Tween-20) supplemented with 1% milk powder for final use. After washing, the blots were incubated with the secondary antibody against rabbit IgG, alkaline phosphatase conjugated from Fermentas (St. Leon-Rot, Germany). Bands were detected with NBT/BCIP solution from Roche (Mannheim, Germany).

2.4. Enrichment of the pyruvate dehydrogenase complex

The PDC purification protocol was adapted from Camp and Randall (1985). Chloroplasts were frozen for 5 min to facilitate breakage before they were osmotically shocked by resuspension in RS-buffer (50 mM Hepes–KOH, pH 8.0; 5 mM $MgCl_2$; 100 mM NaCl). The precipitation steps were conducted with 3% and 6% PEG8000, respectively. The pellet was resuspended in a minimal volume RS-buffer and used in enzymatic assays and for 2D-BN/SDS–PAGE.

Further enrichment was done by glycerol gradient centrifugation. The method was adapted from Camp and Randall (1985). PDC-enriched sample corresponding to 1.5 mg protein was loaded onto a 10 ml linear 10% to 50% (v/v) gradient. Pyruvate was excluded from the gradient solution. The sample was centrifuged for 6 h at 38,000 rpm in a swing-out rotor (SW40Ti, Beckman Coulter, Inc.).

2.5. Enzymatic assays

CO_2 release from [1,2-^{14}C]glycolate or [1-^{14}C]glyoxylate (Hartmann Analytics, Braunschweig, Germany) was measured in disrupted chloroplasts corresponding to 40 μg of plastidal protein or 5 μg enriched PDC, respectively. Specific radioactivities were 1850 MBq $mmol^{-1}$ and final concentrations were 0.1 mM for each substrate. Released CO_2 was absorbed from the gaseous phase. For this, a 0.5 ml reaction tube containing the reaction mix was fixed on the inside of a 5 ml tube containing 1 ml 0.5 M NaOH. Samples were incubated overnight and NaOH was constantly mixed to absorb all released CO_2. For inhibition experiments, 1 mM of either glyoxylate, pyruvate, formate, oxalate or malate, pH 8.0, was added at the start of the assay.

The pyruvate dehydrogenase assay was adapted from Lernmark and Gardestrom (1994). Pyruvate dependent NAD^+ reduction was recorded photometrically at 340 nm in a plate reader (Synergy Mx, Biotek, Winooski, USA) at 25 °C. The assay-medium contained 50 mM Hepes–KOH, pH 8.0; 100 mM NaCl; 5 mM $MgCl_2$; 2 mM NAD^+; 0.2 mM CoA; 0.4 mM TPP (thiamine pyrophosphate); 0.2% w/v BSA and 20 μg disrupted chloroplasts or 3 μg enriched PDC, respectively.

2.6. 2D blue native (BN)/SDS–PAGE and 1D SDS–PAGE

200 μg of precipitated protein resuspended in 100 μl of RS-buffer or isolated chloroplasts in 100 μl SH-buffer corresponding to 400 μg protein (see above) were supplemented with 100 μl solubilization buffer BN (1.5 M aminocaproic acid; 100 mM Bis–tris; 1 mM EDTA, pH 7.0) containing *n*-dodecylmaltoside (Sigma–Aldrich, St. Louis, USA) with a final detergent/protein ratio of 2 g/g and incubated on ice for 10 min. 10 μl of Coomassie blue solution (750 mM aminocaproic acid; 5% w/v Coomassie blue 250 G) were added to each sample prior gel-loading. High-range BN–PAGE was carried out according to Wittig et al. (2006) using a gradient-gel (2.5–16% acrylamide from top to bottom). Tricine SDS–PAGE (Schagger and von Jagow, 1987) for the second gel dimension was carried out in a 3-step gel (16.5% polyacrylamide separation gel; 10% polyacrylamide spacer gel; 2.5% polyacrylamide sample gel; acrylamide-bisacrylamide ratio 16:1, Jansch et al., 1996). 2D gels had dimensions of 16 cm × 16 cm × 0.1 cm and all electrophoretic separations were carried out in the Protean II electrophoresis chamber from Bio-Rad (Hercules, USA). Proteins were stained by the Coomassie blue-colloidal procedure (Neuhoff et al., 1988). Images of the stained gels were acquired using Umax Powerlook III Scanner (Umax Data System, Taipei, Taiwan) with a resolution of 400 dpi. Protein spots of interest were selected by visual inspection of the gel.

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